

**Heme Iron Causes Cytotoxicity to Lung Microvascular Endothelial Cells
through Oxidant Lipid Signaling: Protection by Novel Lipophilic Thiol
Chelator and Antioxidant Drug, *N,N'*-bis-2-Mercaptoethyl Isophthalamide
(NBMI) and Implications in Treatment of Sickle Cell Disease**

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ABSTRACT

Hereditary hemoglobinopathies, mainly sickle cell disease (SCD) and β -thalassemia, contribute to iron overload through hemolysis and hemoglobin (Hb) degradation. Hemin (Fe^{3+} -heme), released from the extracellular Hb, is identified as a critical player in hemoglobinopathies. Heme and iron released from hemoglobin during hemoglobinopathies lead to reactive oxygen species formation (ROS) and oxidant injury to cells and tissues, including the vascular endothelium. Pulmonary hypertension has been recognized as a serious complication in SCD and lung microvasculature appears to be a target to iron-induced lung microvascular endothelial dysfunction contributing to pulmonary hypertension in SCD. Currently available iron chelation therapies for the protection against iron-induced stress in SCD patients are limited and not efficacious. Therefore, here we hypothesized that iron overload would cause reactive oxygen species (ROS) generation and oxidative stress in SCD crisis triggering the redox-regulated activation of the membrane phospholipid-hydrolyzing and cellular lipid signaling enzyme, phospholipase D (PLD) in lung microvascular endothelium, which could be the underlying mechanism(s) of pulmonary hypertension in SCD patients. In our current investigation, we established the oxidant and thiol-redox mediation of iron-induced (hemin) cytotoxicity through PLD-mediated lipid signaling in the bovine lung microvascular endothelial cell (BLMVEC) model *in vitro*. Additionally, our study revealed that hemin caused cytotoxicity through ROS generation, oxidative stress, depletion of glutathione (GSH) redox status, membrane lipid peroxidation, and phospholipase D (PLD) activation, formation of PLD-generated bioactive lipid mediator, phosphatidic acid (PA), and lipid signaling in BLMVECs. The PLD-specific pharmacological inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide hydrochloride hydrate (FIPI), not only attenuated hemin-induced PLD activation but also protected against cytotoxicity

of hemin in BLMVECs suggesting the regulatory role of PLD and the PLD-generated bioactive lipid, PA in cytotoxicity of hemin in endothelial cells (ECs). Furthermore, our current study demonstrated that *N,N'*-bis-2-mercaptoethyl isophthalamide (NBMI), a novel synthetic, non-toxic, and lipophilic thiol-redox heavy metal chelator and antioxidant drug, offered a greater efficacy in attenuating the hemin-induced oxidative stress and PLD activation and protection against cytotoxicity of hemin in lung microvascular ECs as compared to the currently used iron and trace metal chelating drugs and thiol-protectants, thus offering promise for the treatment of iron overload and associated vascular EC injury during hemoglobinopathies including SCD.

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INTRODUCTION

Sickle cell disease (SCD) and β -thalassemia are conditions in which red blood cells (RBCs) contain abnormal hemoglobin (Hb), which results to the inability to effectively transport oxygen and thus leads to anemia in patients. SCD is caused by a substitution of valine for glutamic acid at the β -6 position in the Hb β -chain. This substitution results in production of hemoglobin S (HbS), which polymerizes under deoxygenated conditions (Jaffe and Neumann, 1964). Production of hemoglobin C (HbC) is the result of a mutation at the same β -6 position that leads to intraerythrocytic crystal formation under oxygenated conditions (Hirsch et al., 1985). As a result of the abnormal Hb produced from these mutations, RBCs are degraded through a process known as Hb autoxidation. Under normal physiological conditions, only about 3% of the total body hemoglobin undergoes autoxidation every day (Jaffe and Neumann, 1964). However, since both HbS and HbC are less stable than normal adult hemoglobin (HbA), Hb autoxidation occurs at an accelerated rate in SCD and thalassemia patients.

During Hb autoxidation (degradation), hemoglobin (Hb) bursts from RBCs (hemolysis) releasing naked Hb devoid of its antioxidant counterparts that are normally available within the RBC. Therefore, the release of Hb can cause severe oxidative damage in the vasculature and in exposed tissues (Balla et al., 2007). The process of Hb autoxidation produces toxic degradation products such as hemin (ferric protoporphyrin-IX or Fe^{3+} -heme) (Fig. 1), methemoglobin (methHb), and superoxide anion (O_2^-). To neutralize Hb and its reactive degradation products, specialized plasma scavenger proteins typically sequester the toxic compounds and transport them to isolated compartments where heme-oxygenases can metabolize hemin into less toxic

metabolites. The presence of native thiol-redox antioxidants (glutathione, GSH) (Fig. 2) and antioxidant enzymes (catalase, superoxide dismutase, and GSH peroxidase) also assist in the attenuation of hemin-induced oxidant toxicity by neutralizing superoxide and metHb-induced toxicity. However, when these clearance and detoxifying systems are overwhelmed by large amounts of intravascular hemolysis and autoxidation (due to the presence of destabilizing hemoglobin mutations or chain imbalance as in the case of SCD or β -thalassemia), the excessive amount of hemin produced is able to induce oxidative stress and oxidant injury in cells and tissues (Balla et al., 2007).

In addition, the escalated rate of Hb degradation through Hb autoxidation in SCD patients results in an accumulation of iron (Fe, released during Hb autoxidation) in cell membranes of pathological RBCs and surrounding tissues (Shinar and Rachmilewitz, 1990). Iron is one of the most important trace elements (metals) in the biological systems, serving the essential role in mammalian systems as an important element in several enzymatic reactions while also acting as a crucial element of hemoglobin (Hb) acting as an oxygen carrier for cellular respiration and energy generation. However, iron is also an established Fenton reagent that can conditionally cause severe damage to membrane lipids through formation of ROS leading to lipid peroxidation, which results in adverse effects to cells including cytotoxicity and death. Blood vessels are especially vulnerable to oxidative stress and oxidant injury caused by hemin-overload in SCD (Dominik et al., 2013). The vascular endothelium, the inner lining (monolayer) of the blood vessel, pivotal for the homeostasis of circulation, is also highly vulnerable to oxidative stress caused by iron overload in SCD (Shinar and Rachmilewitz, 1990). Additionally, iron-induced oxidative stress has been established to cause vascular endothelial cell (EC) damage

through oxidant signaling and thiol-redox (GSH) dysregulation. Furthermore, oxidative stress and trace heavy metals have been shown to induce lipid signaling in the cellular membranes through the activation of membrane phospholipid-hydrolyzing enzymes including phospholipases A₂ and D (PLA₂ and PLD) (Mazerik et al., 2007; Secor et al., 2011; Patel et al., 2011). Among the phospholipases, PLD is a ubiquitous enzyme present in mammalian cells including the lung vascular ECs (Parinandi et al., 2001). PLD catalyzes the hydrolysis of membrane phospholipids to form the bioactive signal lipid, phosphatidic acid (PA) which causes EC dysfunction including the lung vascular leak (Fig. 3) (Usatyuk et al., 2013). Also, we have shown that PLD in lung vascular ECs is activated by ROS, oxidants, and thiol-redox dysregulation (Patel et al., 2011; Parinandi et al., 1999). Excessive Hb degradation and RBC hemolysis in SCD can cause vascular and organ dysfunction leading to adverse clinical effects such as hypertension, endothelial dysfunction, renal failure, or immune dysregulation. Pulmonary hypertension is one of the serious complications in SCD patients (Machado and Gladwin, 2005) but the underlying mechanisms are not thoroughly understood. ROS and oxidative stress have been shown to cause lung vascular endothelial damage leading to vascular leak (Usatyuk et al., 2013). However, the Hb degradation product, hemin-induced oxidant lipid signaling that is mediated by PLD has not been reported in the mammalian cellular models including the lung microvascular ECs. **Hence, we hypothesized that iron overload would cause ROS generation and oxidative stress in SCD crisis triggering the redox-regulated activation of the membrane phospholipid-hydrolyzing and cellular lipid signaling enzyme, PLD in lung microvascular endothelium, which could be the underlying mechanism(s) of pulmonary hypertension in SCD patients.**

In order to counteract iron overload encountered in SCD, several iron chelator drugs have been used to treat symptoms of iron toxicity and oxidative stress in SCD patients (Lucania et al., 2011). However, the commonly used iron chelator drugs such as *meso*-dimercaptosuccinic acid (DMSA) and desferal (desferrioxamine) are water-soluble and administered at very high doses and may not reach the lipid-rich membranes which are the target sites of iron-induced oxidative stress (membrane lipid peroxidation). Desferal has also been shown to be toxic and not efficacious to protect against the iron overload in SCD (Galanello et al., 2012). Since the currently available water-soluble chelator drugs are either not effective or marginally effective, a highly efficacious iron chelator is needed to attenuate iron- and heme-Fe-induced oxidant damage in SCD patients. Recently we have reported that our novel and synthetic non-toxic lipid-soluble thiol-redox heavy metal chelator and antioxidant drug *N,N'*-bis-2-mercaptoethyl isophthalamide (NBMI) (Fig. 2) attenuates oxidant- and heavy metal-induced PLD signaling and cytotoxicity in vascular ECs through stabilization of thiol-redox and attenuation of oxidative stress (Secor et al., 2011; Patel et al., 2011). Also, an effective and non-toxic lipophilic iron chelator is highly essential for therapeutic protection against the iron-induced oxidative stress and oxidant injury in the vascular endothelium of the SCD patients. **Therefore, we also hypothesized that our novel lipid-soluble thiol-redox heavy metal chelator and antioxidant drug, NBMI, as opposed to the currently used water-soluble iron chelators (e.g. desferal), can reach the membrane lipid targets of lung vascular ECs (where iron tends to accumulate and cause oxidative stress and lipid signaling events), and thus might offer more effective protection against iron-induced oxidative damage to the lung vascular endothelium.**

In order to test our hypotheses, in the current study, we investigated the role that Fe³⁺-heme (hemin) that arises from Hb degradation in mediating membrane lipid signaling through redox regulation in cultured bovine lung microvascular EC (BLMVEC) model to establish the role of PLD in lung EC dysfunction as a mechanism of pathogenesis of iron overload-driven pulmonary hypertension encountered in SCD complications. Furthermore, we also investigated efficacy of the novel thiol-redox heavy metal chelator and antioxidant drug in attenuating the hemin-induced oxidant PLD signaling and protecting against hemin cytotoxicity in BLMVECs. Our study revealed that hemin caused cytotoxicity through ROS generation, oxidative stress, depletion of glutathione (GSH) redox status, membrane lipid peroxidation, and phospholipase D (PLD) activation, formation of PLD-generated bioactive lipid mediator, phosphatidic acid (PA), and lipid signaling in BLMVECs. Also, our current study demonstrated that the novel thiol-redox heavy metal chelator and antioxidant drug, NBMI, offered a greater efficacy in attenuating the hemin-induced oxidative stress and PLD activation and protection against hemin-induced cytotoxicity in BLMVECs in comparison with the commonly used iron and trace metal chelating drugs and thiol-protectants.

MATERIALS & METHODS

Materials

Bovine lung microvascular endothelial cells (BLMVECs) (passage 2) were purchased from Cell Applications Inc. (San Diego, CA). Phosphate-buffered saline (PBS) was obtained from Biofluids Inc (Rockville, Maryland). Minimal essential medium (MEM), nonessential amino acids, trypsin, FBS, penicillin/streptomycin, Dulbecco modified Eagle medium (DMEM) phosphate-free modified medium, tissue culture reagents, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide reduction kit (MTT assay kit), lactate dehydrogenase cytotoxicity assay kit (LDH release assay kit), and analytical reagents of highest purity were all purchased from Sigma Chemical Co (St Louis, Missouri). Phosphatidylbutanol (PBt), fluorescent C6:0-18:1 NBD-PA, 1-oleoyl-2-{6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino]hexanoyl}-*sn*-glycero-3-phosphate (C18:2 PA), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (C16:0-18:1 PA) were obtained from Avanti Polar Lipids (Alabaster, Alabama). [³²P]orthophosphate (carrier-free) was obtained from New England Nuclear (Wilmington, Delaware). Bleomycin was obtained from Teva Parenteral Medicines (Irvine, California). Desferal was obtained from Calbiochem (San Diego, California). Polyclonal antibody raised against 4-hydroxy-2-nonenal (anti-HNE) was obtained from Enzo Life Sciences (Farmingdale, New York). Endothelial cell growth factor was obtained from Upstate Biotechnology (Lake Placid, New York). Antirabbit AlexaFluor 488-conjugated antibody and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased

from Molecular Probes Invitrogen Co (Carlsbad, California). The novel lipophilic thiol-redox chelator and antioxidant drug and 5-fluoro-2-indolyl des-chlorohalopemide hydrochloride hydrate (FIPI) was prepared as described earlier (Secor et al., 2011; Patel et al., 2011). Glutathione (GSH) assay kit (GSH-Glo) was obtained from Promega Corporation (Madison, Wisconsin). All other reagents were obtained from the Sigma Chemical Company (St. Louis, MO).

In Vitro Cell Culture

BLMVECs were cultured in the minimum essential medium supplemented with 10% FBS, antibiotics, non-essential amino acids, and endothelial cell growth factor up to 90-100% confluence in 35-mm dishes (5×10^5 cells/dish) according to our published procedures (Secor et al., 2011; Patel et al., 2011). BLMVECs were used up to passage 16 for all the experiments.

Lactate Dehydrogenase (LDH) Release Assay of Cytotoxicity

BLMVECs were grown to 90-100% confluence in 24-well culture plates and treated with MEM alone, or MEM containing chosen concentrations of hemin and/or NBMI at varying time points. At the end of the incubation period, the supernatant was removed, and the extent of LDH released was measured by spectrophotometric method according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO) (Hagele et al., 2007; Patel et al., 2012).

Cellular Morphology

BLMVECs were grown in 12-well culture plates to 90-100% confluence. Following their exposure to the chosen concentrations of hemin and/or NBMI or other pharmacological agents at

different time points, the cells were examined under the microscope for morphological alterations as an index of cytotoxicity. Images of cellular morphology were digitally captured using the Zeiss Axiovert S100 at 10x magnification (Hagele et al., 2007; Patel et al., 2012).

Superoxide ($O_2^{\cdot-}$) Measurement by DHE Fluorescence

Generation of superoxide in BLMVECs was determined by DHE (dihydroethidium) fluorescence. BLMVECs grown in 35-mm dishes were treated with different concentrations of hemin and/or NBMI for 1 h. After treatment, the cells were washed once with warm PBS and incubated with DHE (5 μ M) in warm PBS for 30 min. After incubation, cells were washed once with warm PBS and examined under Zeiss LSM 510 Confocal/Multiphoton Microscope at 543-nm excitation with a 560-nm long pass filter at 10X magnification. These images were captured digitally.

4-HNE Staining

BLMVECs grown to 95% confluence in 35-mm dishes (6-well culture plate) on glass coverslips and treated with MEM alone, or MEM containing chosen concentrations of hemin and/or NBMI or MEM-containing and incubated at 37°C in a humidified environment of 5% CO₂-95% air. Cover slips were rinsed three times with PBS-T, and fixed with 3.7% formaldehyde in PBS for 10 min. at room temperature. The cells were permeabilized in 0.25 % Triton X-100 prepared in PBS containing 0.01% Tween-20 (PBS-T) for 5 min. The cells were again washed three times with PBS-T, and treated with PBS-T containing 1 % BSA blocking buffer for 30 min. at room temperature. Cover slips were then incubated overnight at room temperature with the primary antibody [anti-4-hydroxy-2-nonenal (1:150 dilution)] in 1% BSA

solution. After rinsing three times with PBS-T, the cells were labeled with secondary AlexaFluor 488 (1:100 dilution) in 1% BSA in PBS-T for 1 h. Finally, the cells were washed three times with PBS-T, mounted, and examined under Zeiss LSM 510 Confocal/Multiphoton Microscope powered by Argon-2 laser with 500-550 BP filter. The images were captured digitally and the average fluorescent intensity of triplicate samples was determined using ImageJ digital image software.

Phospholipase D Activation in Intact ECs

The activity of PLD in intact BLMVECs was assayed according to our published procedures (Secor et al., 2011; Patel et al., 2011; Sherwani et al., 2013). Phosphatidylbutanol (Pbt) formed from the transphosphatidyl reaction between the PLD-hydrolyzed phosphatidic acid (PA) and the primary alcohol (1-butanol) in the incubation medium was assayed as an index of PLD activity in BLMVECs. BLMVECs grown in 35-mm dishes (5×10^5 cells/dish) were labeled with [32 P]orthophosphate (5 μ ci/mL) in DMEM phosphate-free medium containing 2% (v/v) fetal bovine serum (FBS) for 6-14h. Cells were washed with MEM and incubated at 37°C in 1 mL of MEM containing 0.05% (v/v) 1-butanol in absence and presence of desired concentrations of hemin for different time points under a humidified 95%air – 5% CO₂ atmosphere. In certain cases, ECs were pre-treated for 1 h or 4 h with selected pharmacological inhibitors prior to exposure to hemin. Cells were then either challenged with hemin or co-treated with hemin and pharmacological inhibitors for the desired time points. The incubations were terminated by the addition of 1 mL methanol:HCl (100:1, v/v). Lipids were extracted according to the method of Bligh and Dryer procedure. [32 P]-labeled phosphatidylbutanol ([32 P]Pbt) formed from PLD activation and transphosphatidyl reaction, as an index of PLD activity in

intact cells, was separated by thin-layer chromatography (TLC). Radioactivity associated with the [^{32}P]PBt was quantified by liquid scintillation counting and data were expressed as DPM normalized to 10^6 counts in the total cellular lipid extract or as % of control (vehicle-treated cells).

Glutathione (GSH) determination

Intracellular soluble thiol (GSH) levels were determined using the GSH-Glo assay kit. BLMVECs grown up to 90-100% confluence in 96 well plates were pre-treated with MEM alone or MEM containing desired concentrations of NBMI or NAC for 2 h under a humidified 95% air – 5% CO_2 atmosphere according to our published method (Patel et al., 2012). Following incubation, cells were treated with hemin and NBMI/NAC for 4 h. intracellular GSH levels were determined according to the manufacturer's recommendations (Promega Corp. Madison, WI).

Phosphatidic Acid Localization Confocal Fluorescence Microscopy

BLMVECs grown on sterile coverslips in 35-mm dishes were treated with MEM alone and MEM containing fluorescent $\text{C}_{6:0-18:1}$ NBD-PA (5 $\mu\text{mol/L}$) for 24 hours under a humidified atmosphere of 95% air – 5% CO_2 at 37°C . At the end of the incubation period, cells attached to coverslips were washed with 1x PBS, fixed with 3.7% paraformaldehyde for 10 minutes, permeabilized with 0.25% triton X-100 in Tris buffer saline Tween-20 (TBST) containing 0.01% Tween-20 for 5 minutes, then blocked for 30 minutes with 1% BSA in 0.01% TBST, and incubated for 5 minutes at room temperature with 1% (v/v) DAPI in PBS to visualize the nuclei. The coverslips with cells were then mounted and viewed under a Zeiss Confocal fluorescence microscope at a magnification of 60x, and pictures were captured digitally.

Phosphatidic Acid Formation in Intact ECs

The formation of phosphatidic acid (PA) in intact cells was determined according to our published procedure (Patel et al., 2011). At the end of the experiments, treatments of cells were terminated by the addition of 1 mL methanol:HCl (100:1, v/v). Lipids were extracted essentially according to the method of Bligh and Dyer, as described previously. [³²P]-labeled PA formed from PLD activation, as an index of PLD activity in intact cells, was separated by TLC. Autoradiogram was developed from the TLC plate with [³²P]PA separated from other phospholipids. Intensity of the radioactivity associated with the [³²P]PA was quantified by liquid scintillation counting as described previously.

Protein Determination

Cellular protein was determined by the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, Illinois) according to the manufacturer's recommendations.

Statistical Analysis

All experiments were done in triplicate. Results were expressed as mean \pm standard deviation (SD). Statistical analysis of data was done by one-way analysis of variance (ANOVA) using the SigmaStat (Jandel Scientific, San Rafael, California). The level of statistical significance was taken as $P \leq 0.05$.

Preparation of Solutions Containing Pharmacological Agents for Treatment of Cells

The stock solutions of lipophilic pharmacological agents including NBMI and FIPI were freshly prepared in DMSO and diluted in MEM for treatment of cells. The final DMSO concentration in the cell treatment medium was 0.1% (vol/vol) which did not appear to have any effect on the outcome of the experiments. All other solutions of water-soluble pharmacological compounds were freshly prepared in MEM for treatment of cells.

RESULTS

Hemin induces PLD activation in dose- and time-dependent fashion in BLMVECs. Iron overload and oxidative stress have been reported as the underlying mechanisms of SCD complications including inflammation, pain, and vascular dysfunctions (Lucania et al., 2011; Machado and Gladwin, 2010; Belcher et al., 2010). Iron and heme are known to cause oxidative stress through the generation of ROS (Belcher et al., 2010). Earlier, we have reported that ROS and oxidants induce PLD activation in vascular ECs (Parinandi et al., 1999 & 2001). Therefore, here we investigated to demonstrate whether hemin would induce PLD activation in BLMVECs. As shown in Fig. 3, PLD activity was measured as the extent of formation of [32 P] phosphatidylbutanol (PBt) in intact cells. Hemin caused significant activation of PLD in dose- and time-dependent manner at 50-100 μ M concentration at 1 and 4 h (Fig. 4A) as compared to that in the control untreated cells. At 4 h of treatment, the hemin-induced activation of PLD was 3-fold at 50 μ M dose as compared to that in the cells treated with 50 μ M hemin for 1 h (Fig. 4A). However, at 100 μ M concentration, hemin significantly caused nearly 3.5-fold and 5-fold increase in PLD activation in BLMVECs as compared to that in the control untreated cells (Fig. 4A). When BLMVECs were treated with hemin (10, 25, and 50 μ M) for 6 h, a significant and dose-dependent induction of PLD activation was observed showing nearly a 15-fold increase in

the enzyme activation at 50 μ M dose of hemin as compared to the same in the control untreated cells (Fig. 4B). A time-course study at 2, 4, and 6 h showed that hemin at 50 μ M concentration caused a clear time-dependent and significant induction of PLD activation in BLMVECs as compared to that in the control untreated cells (Fig. 4C). These results clearly demonstrated that hemin induced both dose- and time-dependent activation of PLD in BLMVECs. Furthermore, 50 μ M concentration of hemin and 6 h treatment time were chosen as the optimal dose of hemin and treatment time, respectively for PLD activation in BLMVECs in the subsequent experiments of this study.

Hemin induces formation of PLD-generated bioactive lipid, PA in dose-dependent fashion in BLMVECs. As the previous experiments only demonstrated the hemin-induced activation of PLD in both dose- and time-dependent fashion in BLMVECs as measured by the enzyme activity in cells, here we investigated hemin-induced formation of PLD-generated bioactive lipid, PA in intact BLMVECs. Hemin induced formation of PA in a dose-dependent manner (10-50 μ M) in BLMVECs as determined by the [32 P] labeling of cells and TLC analysis of lipids following the treatment of cells with hemin as compared to that in the control untreated cells (Fig. 5A). Quantification of radioactivity [32 P] in the PA separated by TLC clearly showed significant dose-dependent formation of PA in BLMVECs induced by hemin (Fig. 5B). At 50 μ M concentration, hemin at 6 h of treatment, caused ~4.5-fold increase in PA formation in BLMVECs. These results showed that hemin induced formation of the PLD-generated bioactive lipid, PA in BLMVECs.

Antioxidants modulate hemin-induced PLD activation in BLMVECs. Earlier, we have reported that antioxidants attenuate oxidant-induced PLD activation in ECs (Parinandi et al., 1999; Patel et al., 2011). Therefore, here we investigated whether the commonly used antioxidants such as vitamin C, propyl gallate, and Trolox (water-soluble form of vitamin E) would attenuate hemin-induced PLD activation in BLMVECs. Vitamin C alone (50 and 500 μ M and 1 mM), in a dose-dependent fashion, induced significant PLD activation at 6 h of treatment as compared to the same in the control untreated cells (Fig. 6A). Also, vitamin C caused a synergistic effect of significantly enhancing the hemin-induced PLD activation in a dose-dependent fashion as compared to the same in the hemin-treated cells alone at 6 h of treatment (Fig. 6A). On the other hand, the phenolic antioxidant, propyl gallate did not alter hemin-induced PLD activation in BLMVECs at 50 and 100 μ M doses, but significantly enhanced hemin-induced activation of the enzyme at 500 μ M dose following 6 h of treatment of cells (Fig. 6B). However, the water-soluble form of vitamin E (α -tocopherol), an established lipid-soluble antioxidant at 500 μ M and 1 mM doses, caused significant attenuation of hemin-induced PLD activation at 6 h of treatment in BLMVECs (Fig. 6C). Overall, this study demonstrated that among the three tested antioxidants (vitamin C, propyl gallate, and Trolox), only Trolox effectively attenuated hemin-induced PLD activation in BLMVECs.

Sulfhydryl agents and thiol-protectants attenuate hemin-induced PLD activation in BLMVECs. Oxidants are known to alter or deplete the thiol-redox status in cells (Parinandi et al., 1999; Patel et al., 2012). Also, our earlier studies have shown that oxidant-induced PLD activation is thiol-redox-regulated and sulfhydryl agents including the thiol-protectants attenuate oxidant-induced PLD activation in ECs (Parinandi et al., 1999). Hence, here we investigated

whether the widely used sulfhydryl agents and thiol-protectants would attenuate hemin-induced activation of PLD in BLMVECs. The cellular soluble tripeptide thiol, GSH, offered significant attenuation of hemin-induced PLD activation at 500 μ M and 1 mM doses at 6 h treatment of cells as compared to the same in the cells treated with hemin (50 μ M) alone (Fig. 7A). On the other hand, DTT, another thiol-protectant caused a significant and marked attenuation of hemin-induced PLD activation in a dose-dependent fashion as compared to the enzyme activation in the cells treated with hemin (50 μ M) alone for 6 h (Fig. 7B). Although DTT alone at 1 mM dose caused PLD activation in BLMVECs at 6 h of exposure, the same caused significant and nearly complete attenuation of hemin-induced PLD activation to the extent exhibited by the control untreated cells (Fig. 7B). NAC, the commonly used thiol-protectant and thiol enhancer, at both 50 μ M and 1 mM concentrations, failed to cause attenuation of hemin-induced PLD activation in BLMVECs at 6 h of treatment, whereas the novel lipid-soluble thiol metal-chelator and antioxidant drug, NBMI, offered significant attenuation of hemin-induced activation of PLD in cells under identical conditions (Fig. 7C). The two other tested sulfhydryl agents such as L-cysteine and L-cysteamine even at 1 mM dose were ineffective in causing attenuation of hemin-induced PLD activation in BLMVECs (Figs. 7D and 7E). Altogether, these results revealed that the sulfhydryl agents and thiol protectants including GSH and DTT significantly attenuated hemin-induced PLD activation in BLMVECs suggesting the role of cellular thiols in regulation of PLD activation by hemin.

Heavy metal-chelating agents and NBMI attenuate hemin-induced PLD activation in BLMVECs. Trace metals have been shown to activate phospholipases including PLA₂ and PLD in vascular ECs (Natarajan et al., 1993; Hagele et al., 2007; Mazerik et al., 2007; Secor et al.,

2011; Sherwani et al., 2013). Earlier, we have reported that heavy metal chelators and the novel lipophilic thiol-redox chelator, NBMI attenuates mercury-induced PLD activation in vascular ECs (Secor et al., 2011). Therefore, here we investigated the efficacies of several commonly used trace metal chelators including desferal, DMSA, EDTA, PDTC, and NBMI in attenuating hemin-induced PLD activation in BLMVECs. PDTC, a dithiocarbamate heavy metal chelator was shown to cause synergistically and significantly enhanced hemin-induced PLD activation in a dose-dependent fashion in BLMVECs that were treated with hemin (50 μ M) for 6 h and the chelator drug as compared to the cells treated with hemin alone under identical conditions (Fig. 8A). NBMI (50 μ M) caused significant attenuation of hemin-induced PLD activation in BLMVECs as compared to the same in the cells treated with hemin (50 μ M) alone for 6 h (Fig. 8B). DMSA (50 μ M and 1 mM) did not appear to be effective in causing attenuation of hemin-induced PLD activation in BLMVECs (Fig. 8C). EDTA was observed to cause a marginal but significant extent of attenuation of hemin-induced PLD activation in BLMVECs (Fig. 8D). However, NBMI (50 μ M), in comparison with DMSA (50 μ M and 1 mM) and EDTA (50 μ M and 1 mM), exhibited greater and significant efficacy in attenuating hemin-induced PLD activation in BLMVECs (Figs. 8C and 8D). However, the widely used iron chelator, desferal, offered significant and dose-dependent attenuation hemin-induced PLD activation at 50 μ M and 1 mM doses in BLMVECs treated with hemin (50 μ M) for 6 h (Fig. 8E). Furthermore, desferal at 1 mM concentration was required to achieve the same and significant extent of attenuation of hemin-induced PLD activation in BLMVECs that was caused by NBMI at the dose of 50 μ M (Fig. 8E). These results clearly demonstrated that among the widely used trace metal chelators including EDTA and desferal were less effective than the novel lipophilic thiol-redox heavy metal chelator NBMI in attenuating hemin-induced PLD activation. Furthermore, NBMI at 50

μM concentration was effective in causing significant and marked attenuation of hemin-induced PLD activation in BLMVECs while mM concentrations of EDTA and desferal were required to achieve the same.

NBMI attenuates hemin-induced formation of PLD-generated bioactive lipid, PA in BLMVECs. The earlier experiments of the current study revealed that NBMI caused a significant and marked attenuation of hemin-induced activation of PLD in BLMVECs. Hence, here we investigated whether NBMI would attenuate hemin-induced formation of PLD-generated bioactive lipid, PA, in BLMVECs. As shown in Fig. 9A, the TLC analysis of lipids of cells treated with NBMI (50 μM) clearly revealed the attenuation of formation of PA in cells treated with hemin (50 μM) for 6 h. Quantification of [^{32}P] radioactivity in the PA of cell membrane lipids showed significant and marked attenuation of hemin-induced formation of PLD-generated PA in BLMVECs under identical conditions (Fig. 9B). These results demonstrated that NBMI was effective in causing attenuation of formation of PLD-generated bioactive lipid, PA, in BLMVECs.

PLD-specific inhibitor, FIPI attenuates hemin-induced PLD activation in BLMVECs.

Earlier, we have reported that the novel PLD-specific inhibitor, FIPI attenuates oxidant and heavy metal-induced PLD activation in vascular ECs (Patel et al., 2011; Secor et al., 2011). Therefore, here we investigated whether FIPI could attenuate hemin-induced activation of PLD in BLMVECs. Our results revealed that the PLD-specific inhibitor, FIPI caused dose-dependent (0.1, 0.5, and 1 μM) and significant inhibition of PLD activation in BLMVECs treated with hemin (50 μM) for 6 h (Fig. 10). Also, these results confirmed that the PBt formation induced by

heme in BLMVECs was mediated by PLD as the PLD-specific inhibitor caused marked attenuation of the PLD-mediated PBt formation in cells.

NBMI protects against hemin-induced ROS formation and loss of GSH in BLMVECs.

Trace metals including iron have been established as crucial players in causing ROS generation and loss of thiol-redox and GSH status in vascular ECs (Hagele et al., 2007; Patel et al., 2012). Furthermore, we have reported that NBMI attenuates oxidant-induced ROS production and loss of GSH in vascular ECs (Patel et al., 2012). Therefore, here we investigated whether hemin would induce ROS generation and loss of GSH in BLMVECs and NBMI would be effective in attenuating those outcomes in BLMVECs. As shown in Fig. 11A, hemin (50 μ M) caused marked increase in ROS ($O_2^{\cdot-}$) generation at 1 h of treatment of BLMVECs as measured by the intracellular fluorescence of the $O_2^{\cdot-}$ -specific intracellular fluorophore which was attenuated by NBMI (50 μ M). Also, the intracellular GSH analysis revealed that hemin (10, 25, and 50 μ M) caused a drastic and significant loss of GSH in a dose-dependent fashion in cells following their treatment with hemin for 4 h which was significantly attenuated by NBMI (50 μ M) (Fig. 11B). Moreover these results revealed that basal GSH levels in cells treated with NBMI (50 μ M) alone were also elevated by 40-fold and the protection against hemin-induced loss of GSH in BLMVECs diminished with the increase in the dose of hemin. Overall, these results demonstrated that hemin caused ROS generation and loss of cellular GSH in BLMVECs which were attenuated by NBMI.

NBMI protects against hemin-induced lipid peroxidation in BLMVECs. Oxidants and trace metals have been shown to cause lipid peroxidation which is a hallmark of oxidative stress in

vascular ECs (Balla et al., 2005; Maytin et al., 1999; Valko et al., 2005; Jomova and Valko, 2011). Also, in the earlier experiments of this study, it was revealed that hemin-induced ROS production and loss of cellular GSH was attenuated by NBMI. Furthermore, trace metal chelators and antioxidants have been shown to protect against oxidant- and metal-induced lipid peroxidation (Aviram et al., 1998). Therefore, here we investigated whether (i) hemin would induce lipid peroxidation in BLMVECs and (ii) the novel lipophilic thiol-redox chelator and antioxidant drug, NBMI would attenuate hemin-induced lipid peroxidation in BLMVECs. The confocal immunofluorescence microscopy of formation and intracellular localization of 4-hydroxy-2-nonenal (4-HNE, an electrophilic product of membrane phospholipid-esterified polyunsaturated fatty acid peroxidation) revealed marked and significant induction of lipid peroxidation in BLMVECs treated with hemin (50 μ M) for 1 h (Figs. 12A and 12B). Furthermore, NBMI (50 μ M) was observed to significantly and markedly attenuate hemin-induced lipid peroxidation in BLMVECs. These results demonstrated that the novel lipophilic thiol-redox chelator and antioxidant drug was effective in attenuating hemin-induced membrane lipid peroxidation in BLMVECs.

NBMI protects against hemin-induced cytotoxicity in BLMVECs. Oxidants and trace metals have been shown to cause cytotoxicity in vascular ECs (Secor et al., 2011; Patel et al., 2012). Also, the novel lipophilic thiol-redox heavy metal chelator and antioxidant drug has been shown to protect against oxidant- and trace metal-induced cytotoxicity in vascular ECs (Secor et al., 2011; Patel et al., 2012). Therefore, here we investigated whether (i) hemin would cause cytotoxicity in BLMVECs and (ii) NBMI would offer protection against hemin-induced cytotoxicity in BLMVECs. Hemin (10 and 25 μ M) caused alterations in cell morphology (e.g.

rounding of cells and loss of shape as an indices of cytotoxicity) at 4 h of treatment which was completely protected by NBMI (50 μ M) treatment (Fig. 13A). Hemin (50 μ M) at 6 h of treatment was also observed to cause significant and marked cell membrane damage as evidenced by the release of LDH into the medium, which serves as the physiological and biochemical index of cytotoxicity (Fig. 13B). NBMI, only at the concentration of 50 mM was shown to exhibit protection against hemin-induced LDH release from BLMVECs under identical conditions (Fig. 13B). On the contrary, NAC, the common thiol-protector and enhancer appeared to significantly enhance hemin-induced LDH release (cytotoxicity) at 50 μ M dose and did not alter the cytotoxic response at 1 mM dose whereas NBMI at 50 μ M dose exhibited significant and marked protection against hemin-induced LDH release from BLMVECs (Fig. 13C). These results clearly demonstrated that hemin induced cytotoxicity in BLMVECs, which was protected by the novel thiol-redox chelator and antioxidant drug, NBMI but not by the widely used thiol-protector and enhancer, NAC.

PLD-specific inhibitor, FIPI protects against hemin-induced cytotoxicity in BLMVECs.

Our earlier studies have shown that the novel PLD-specific inhibitor, FIPI not only attenuates oxidant and trace metal-induced PLD activation but also protects against cytotoxicity induced by oxidants and trace metals in vascular endothelial cells (Secor et al., 2011; Patel et al., 2011). Also, in the earlier experiments of the current study demonstrated that FIPI significantly attenuated hemin-induced PLD activation in BLMVECs. Therefore, here we investigated whether the PLD-specific inhibitor, FIPI, would protect against hemin-induced cytotoxicity in BLMVECs. Hemin (50 μ M) caused marked alterations of cell morphology (as an index of cytotoxicity) in BLMVECs at 4 h of treatment and FIPI (0.1 and 0.5 μ M) distinctly protected

against hemin-induced alterations of cell morphology (Fig. 14A). Additionally, hemin (50 μ M) at 6 h of treatment caused significant cell membrane damage and release of intracellular LDH into the medium as compared to that in the control untreated cells that was significantly attenuated by FIPI (0.1, 0.5, and 1.0 μ M) in a dose-dependent fashion as compared to the cells treated with hemin alone under identical conditions (Fig. 14B). These results clearly demonstrated that the PLD-specific inhibitor FIPI protected against hemin-induced cytotoxicity in BLMVECs confirming the role of PLD therein.

DISCUSSION

The present study revealed that the hemoglobin degradation product, hemin that accumulates during SCD crisis caused ROS generation, oxidative stress, loss of cellular GSH redox status, activation of PLD, and formation of intracellular bioactive lipid signal mediator (PA) leading to cytotoxicity in cultured lung vascular EC model. The hemin-induced EC damage was protected by certain sulfhydryl agents, thiol-protectants, and especially by the novel synthetic thiol-redox heavy metal chelator and antioxidant drug, NBMI with a greater efficacy. Furthermore, the current study also demonstrated that the novel lipophilic PLD-specific

pharmacological inhibitor, FIPI, attenuated hemin-induced PLD activation and protected against hemin-induced cytotoxicity in BLMVECs suggesting that hemin-induced cytotoxicity in ECs was mediated by the redox-regulated PLD lipid signaling through the generation and action of the bioactive lipid signal mediator, PA (Fig. 15).

PLD is a ubiquitous enzyme present in the mammalian cells including the vascular ECs in two different isoforms as PLD₁ and PLD₂ (Cummings et al., 2002; Parinandi et al., 2001). Although the cellular localization of both isoforms is not known precisely, PLD₁ has been localized predominantly in the nucleus whereas PLD₂ has been detected in both cytosolic and nuclear compartments of the vascular ECs (Parinandi unpublished work). PLD₁ is known to be activated by co-factors including the adenosine 5'-diphosphate ribosylation factor (ARF) and phosphatidylinositol bisphosphate (PIP₂), whereas PLD₂ is activated by PIP₂ (Parinandi et al., 2001; Natarajan et al., 2001). Protein kinases such as the protein kinase C (PKC), protein tyrosine kinases (PTyKs) including Src kinase, and the mitogen-activated protein kinases (MAPKs) including the p38 MAPK and extracellular signal-regulated kinases (ERK1/2) have been identified to regulate the activation of PLD during conditions of oxidative stress in vascular ECs (Parinandi et al., 2001; Natarajan et al., 2001; Secor et al., 2011). Also, the small G proteins have been shown to regulate the activity of cellular PLDs (Parinandi et al., 2001). In addition, PLD in vascular ECs has been shown to be activated by oxidants, heavy metals like mercury, and perturbations of the thiol-redox (GSH) status in vascular ECs (Natarajan et al., 2001; Parinandi et al., 1999; Secor et al., 2011; Patel et al., 2011). Along these lines, the current investigation clearly demonstrated the oxidant-mediated and thiol-redox-regulated activation of PLD and generation of the bioactive lipid signal mediator (PA) in BLMVECs exposed to hemin, the

regulation of PLD by other signaling protein kinases such as PKC, PTyKs, and MAPKs and small G proteins has not been ruled out. The current work also suggests that heme-iron such as hemin can possibly activate PLD during SCD iron overload leading to lipid signaling cascades in vascular ECs mediated by PLD-generated bioactive lipid signal mediator, PA.

PLD has emerged as an important cellular signaling phospholipase which mediates several cellular functions such as mitogenesis, cytoskeletal regulation, and secretion in several mammalian cells including the vascular ECs (Parinandi et al., 2011). The PLD-generated bioactive lipid signal mediator, PA, can further undergo metabolic transformation into more potent bioactive lipid signal mediators such as the lysophosphatidic acid (LPA; generated by either phospholipase A₁ or A₂) and diacylglycerol (DAG; generated by lipid phosphate phosphohydrolase) (Parinandi et al., 2011). All these bioactive lipid signal mediators generated by PLD are potent signal mediators that affect the target proteins leading to modulation/alteration of cellular functions such as cytoskeletal reorganization, cellular adhesion, mitogenesis, and cell viability. DAG is a known endogenous activator of PKC which mediates several cellular signaling cascades. Also, the signaling actions of LPA are mediated by specific LPA receptors in cells. Recently, we have reported that mercury-induced cytotoxicity in vascular ECs has been regulated by PLD activation through ROS production, oxidative stress, thiol-redox dysregulation, and generation of the bioactive lipid signal mediator, PA (Patel et al., 2011; Secor et al., 2011). We have also reported that PLD regulates the oxidant-induced EC barrier dysfunction and vascular endothelial permeability suggesting that PLD plays a crucial role in mediating the vascular leakiness through oxidative stress mechanism (Usatyuk et al., 2013). These studies support the current findings that hemin-induced PLD through generation and

actions of the bioactive lipid signal mediators (PA, LPA, and DAG) might be responsible for the hemin-induced cytotoxicity in BLMVECs through oxidant regulation. Although no attempts were made in the current study to determine the levels of LPA and DAG in BLMVECs during hemin exposure, it could be surmised that hemin could induce the formation of these bioactive lipid signal mediators that are capable of mediating adverse effects of hemin in lung vascular ECs as the underlying mechanisms of pulmonary hypertension in SCD. Also, this signifies the pressing need for identification and determination of the PLD-generated bioactive lipid mediators as biomarkers of vascular endothelial dysfunction in hemoglobinopathies such as SCD with iron overload.

Hemolysis has been shown to cause release of Hb into extracellular regions causing complications in patients through the release of hemin from Hb (Schaer et al., 2013). Hemin is a potent signal mediator that causes damaging effects in pathological conditions including SCD. Hemin when administered into sickle mice has been shown to cause acute chest syndrome, the hallmark of SCD (Ghosh et al., 2013). Hemin release has been shown to arise from HbS denaturation which accumulates up to 3- to 5-fold in SCD patients suggesting the erythrocyte damage caused by hemin in SCD condition (Liu et al., 1988). Hemin accumulation in SCD pathology has also been suggested to cause erythrocyte damage (Shaklai et al., 1985). Hemin by nature is hydrophobic/lipophilic and tightly associated with the cell membrane (Belcher et al., 2010). Upon reaction with hydrogen peroxide, hemin releases the free redox-active iron which mediates the generation of ROS that cause oxidative stress through peroxidation of membrane lipids and oxidative degradation of proteins and nucleic acids leading to adverse effects on cells under pathophysiological conditions of SCD (Belcher et al., 2010). Thus, hemin is a potent

oxidant that causes damage of cells including the vascular ECs as witnessed in the current study. Although, the exact mechanism of ROS generation induced by hemin in BLMVECs in the current study was not established, the role of endothelial NAD[P]H oxidase in hemin-induced ROS generation in lung microvascular ECs is not ruled out since the role of NAD[P]H oxidase has been shown in ROS generation in lung ECs has been established (Parinandi et al., 2003). Hence, there could be substantial generation of hydrogen peroxide in BLMVECs exposed to hemin which further could have contributed to the release of free iron capable of catalyzing the Fenton reaction in the cells. Thus, the role of hemin-generated free iron in causing oxidative stress in lung vascular ECs appears to be possible.

Pulmonary hypertension, a common SCD complication is being identified as a major risk factor for mortality among adult patients (Machado and Gladwin, 2005). Although several causative factors have been addressed in the pathogenesis of SCD pulmonary hypertension, the role of iron, ROS, oxidative stress and redox signaling leading to lung vascular damage have been emerging (Wong et al., 2012 & 2013; Touyz 2004). In spite of lack of thorough understanding of the specific mechanism(s) of pulmonary hypertension, iron-mediated protein carbonylation has been suggested to play a crucial role in the pathogenesis of pulmonary hypertension (Wong et al., 2013). The NAD[P]H oxidase family of enzymes (Nox) present in the lung vessels have been implicated as the crucial enzymes responsible for ROS generation responsible for pulmonary hypertension (Freund-Michel et al., 2013). Along these lines it is surmised that hemin-induced ROS and oxidative stress in lung vascular endothelium could be responsible for the pathogenesis of pulmonary hypertension in SCD patients.

Iron, whether free iron or Fe^{3+} -heme (hematin), appears as the upstream player to induce ROS generation, oxidative stress, and thiol-redox dysregulation which trigger the down-stream signaling events such as PLD activation and bioactive lipid mediator generation that cause lung vascular endothelial dysfunction and damage. Antioxidants that neutralize ROS and diminish oxidative stress have been recommended as therapeutic agents for pulmonary hypertension (Wong et al., 2013). On the other, iron chelation has been shown to decrease remodeling of pulmonary vasculature indicating that iron-mediated ROS play a mechanistic role in the pathogenesis of pulmonary hypertension (Wong et al., 2012). Therefore, iron chelators appear as promising therapeutic agents in the treatment of SCD pulmonary hypertension. Although the iron chelators such as deferasirox (DFRA), deferoxamine, and deferiprone have been used for iron chelation therapy in SCD patients, these chelators are either not completely efficacious or their safety is questionable (Vlachaki et al., 2013; Lucania et al., 2011; Maggio, 2007). The current study clearly demonstrated that the novel lipophilic thiol-redox heavy metal chelator and antioxidant drug, NBMI, effectively attenuated hemin-induced ROS production, oxidative stress, oxidant lipid signaling, and cytotoxicity in lung vascular ECs (Fig. 15). These results emphasized the advantages of using NBMI as the iron chelator of drug of choice to treat iron overload and iron-mediated SCD complications including pulmonary hypertension for reasons such as (1) NBMI is lipophilic and non-toxic and unlike the water-soluble iron chelator drugs that are commonly used in clinical settings, NBMI can reach the membrane lipid targets protecting against iron-induced oxidative stress by complexing with iron and rendering it catalytically inactive; (2) NBMI has a thiol-stabilizing action and therefore will protect against the loss or dysregulation of thiol-redox status induced by iron; and (3) NBMI also exhibits antioxidant actions and thus protects cells against oxidative stress including lipid peroxidation.

NBMI has dicarboxybenzoate moiety which is naturally present in fruits and bound to two cysteamines (Secor et al., 2011). Hence, it is expected that NBMI can act as both a trace metal (iron) chelator and free radical scavenger. The heavy metal-chelating ability of NBMI has been reported (Secor et al., 2011). Therefore, the EC protective action of NBMI against the hemin-induced cytotoxicity as demonstrated in the current study could have been mediated by the ability of NBMI to stabilize or protect the cellular thiol redox, attenuate oxidative stress, and chelating iron. Moreover, the lipophilicity/hydrophobicity of NBMI could have been advantageous for compound to precisely localize in the hydrophobic phospholipid domains of the EC membrane where the redox signaling events such as the PLD-generated bioactive lipids could operate leading to the hemin-induced cytotoxicity of lung microvascular ECs. The novel lipophilic bifunctional chelating drug, NBMI, with thiol-redox antioxidant action appears to immensely impact the progressing therapies effective for the treatment of iron overload in SCD. Currently available treatments for iron toxicity and overload only include the water-soluble iron chelators and low-molecular weight thiols. On the other hand, the lipophilic drugs including NBMI and FIPI are promising to be more efficacious to offer protection against iron-induced oxidative stress and cell damage, especially those are encountered in SCD through their actions in the hydrophobic lipid bilayer of the cellular membranes that hosts the cellular redox reactions, phospholipid hydrolysis, and oxidant-mediated signaling cascades. Therefore, NBMI appears to be a promising dual action drug capable of iron chelation and antioxidant action that can be used in iron-induced SCD complications including pulmonary hypertension.

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LIST OF ABBREVIATIONS

4-HNE: 4-hydroxy-2-nonenal

ANOVA: analysis of variance

BCA: bicinchoninic acid

BLMVECs: bovine lung microvascular endothelial cells

DAG: diacylglycerol

DAPI: 4',6-diamidino-2-phenylindole dihydrochloride

DFRA: deferasirox

DHE: dihydroethidium

DMEM: Dulbecco's modified Eagle medium

DMSA: *meso*-dimercaptosuccinic acid

DMSO: dimethylsulfoxide

DTT: dithiothreitol

ECs: endothelial cells

EDTA: ethylenediaminetetraacetic acid

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

FIPI: 5-fluoro-2-indolyl des-chlorohalopemide hydrochloride hydrate

GSH: glutathione

Hb: hemoglobin

HbA: adult hemoglobin

HbC: hemoglobin C

HbS: hemoglobin S

LDH: lactate dehydrogenase

LPA: lysophosphatidic acid

MAPK: mitogen-activated protein kinase

MEM: minimal essential medium

metHb: methemoglobin

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NAC: N-acetyl-L-cysteine

NBMI: *N,N'*-bis-2-mercaptoethyl isophthalamide

Nox: NAD[P]H oxidase

PA: phosphatidic acid

PBS: phosphate-buffered saline

PBS-T: phosphate-buffered saline-Tween-20

PBt: phosphatidylbutanol

PDTC: pyrrolidine dithiocarbamate

PIP₂: phosphatidylinositol bisphosphate

PKC: protein kinase C

PLA₂: phospholipase A₂

PLD: phospholipase D

PTyKs: protein tyrosine kinases

RBCs: red blood cells

ROS: reactive oxygen species

SCD: sickle cell disease

TLC: thin-layer chromatography

Figure 1

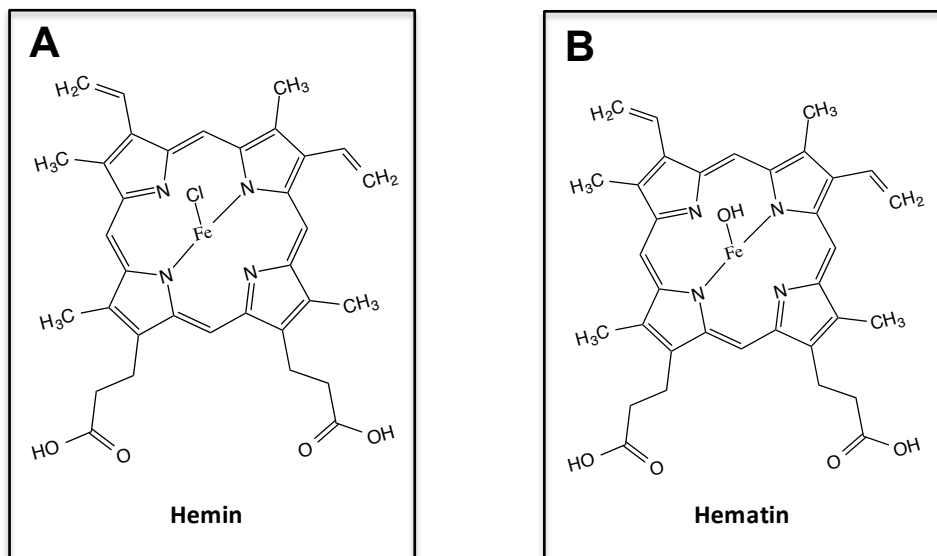


Figure1: Chemical structure of Hemin and Hematin.

Figure 2

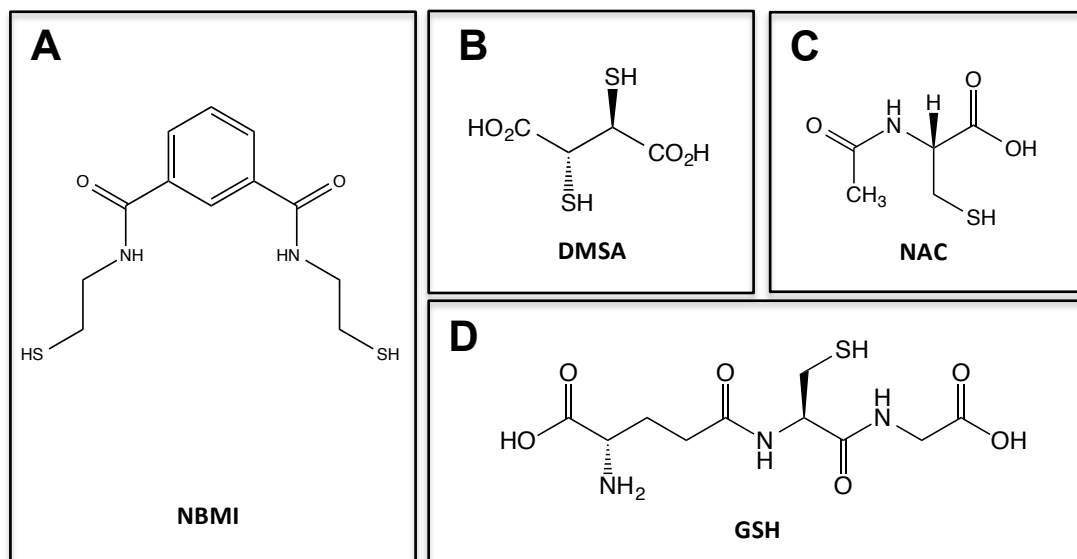


Figure 2: Chemical structure of the novel synthetic lipophilic thiol-redox heavy metal chelator [*N,N'*-bis-2-mercaptoethyl isophthalamide (NBMI), pharmacological compounds N-acetyl-L-cysteine (NAC), meso-2,3-dimercaptosuccinic acid (DMSA), and antioxidant glutathione (GSH).

Figure 3

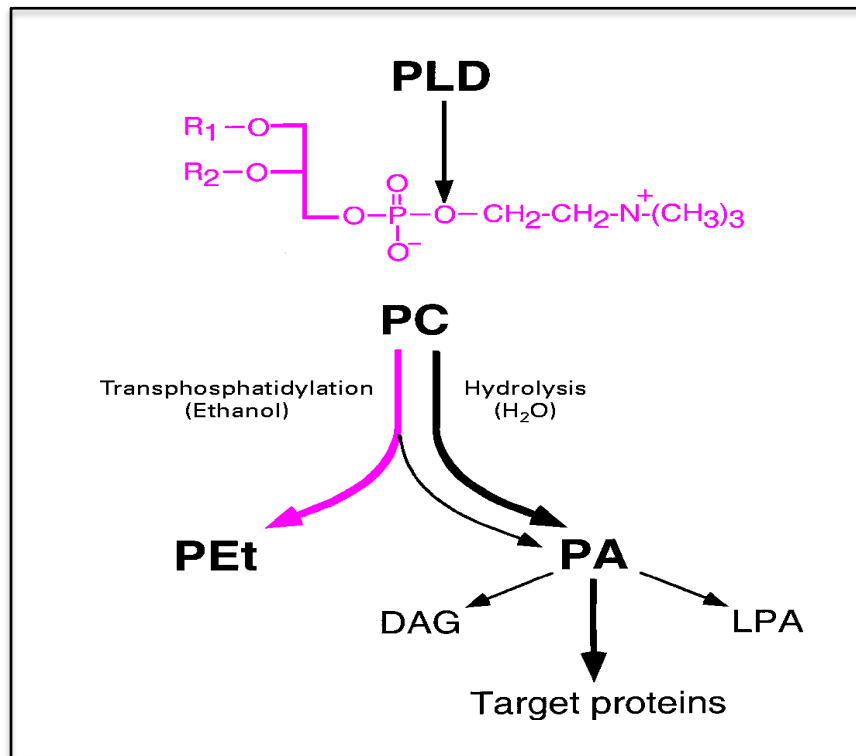


Figure 3: PLD catalyzes the hydrolysis of head group of membrane phospholipids (e.g. phosphatidylcholine, PC) and generates free head group (e.g. choline) and phosphatidic acid (PA). PLD is also capable of using primary alcohols (e.g. ethanol and butanol) to form phosphatidylalcohols (e.g. phosphatidylethanol, PEt and phosphatidylbutanol, PBt). PA can also be metabolized by cells into other bioactive lipids such as diacylglycerol (DAG) and lysophosphatidic acid (LPA). The bioactive lipids such as PA, DAG, and LPA cause cellular signaling.

Figure 4

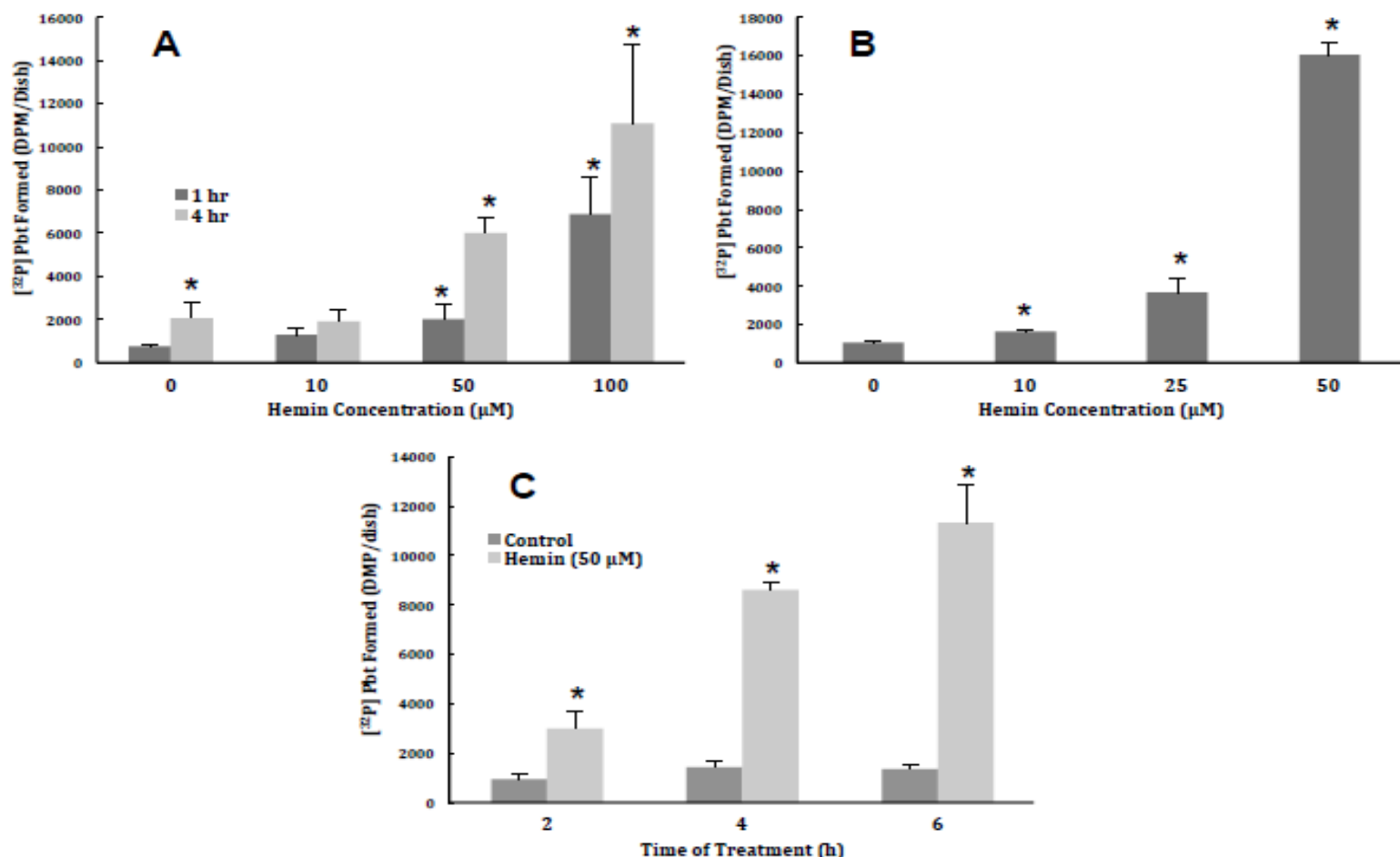


Figure 4: Hemin-induces phospholipase D (PLD) activation in BLMVECs in a dose - and time - dependent fashion. BLMVECs (5×10^5 cells/35 mm dish) were labeled with $[^{32}\text{P}]\text{orthophosphate}$ in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 h. (A) Following $[^{32}\text{P}]\text{orthophosphate}$ labeling, cells were treated with minimal essential medium (MEM) alone or MEM containing varying concentrations of Hemin (10, 50, and 100 μM) for 1 or 4 h. (B) Cells were treated with MEM alone or MEM containing varying concentrations of Hemin (10, 25, 50 μM) for 6 h. (C) Cells were treated with minimal essential medium (MEM) alone or MEM containing Hemin (50 μM) for 2, 4, or 6 h. At the end of the incubation periods, $[^{32}\text{P}]\text{phosphatidylbutanol}$ ($[^{32}\text{P}]\text{Pbt}$) formation was determined and quantified as DPM/dish. Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with MEM alone.

Figure 5

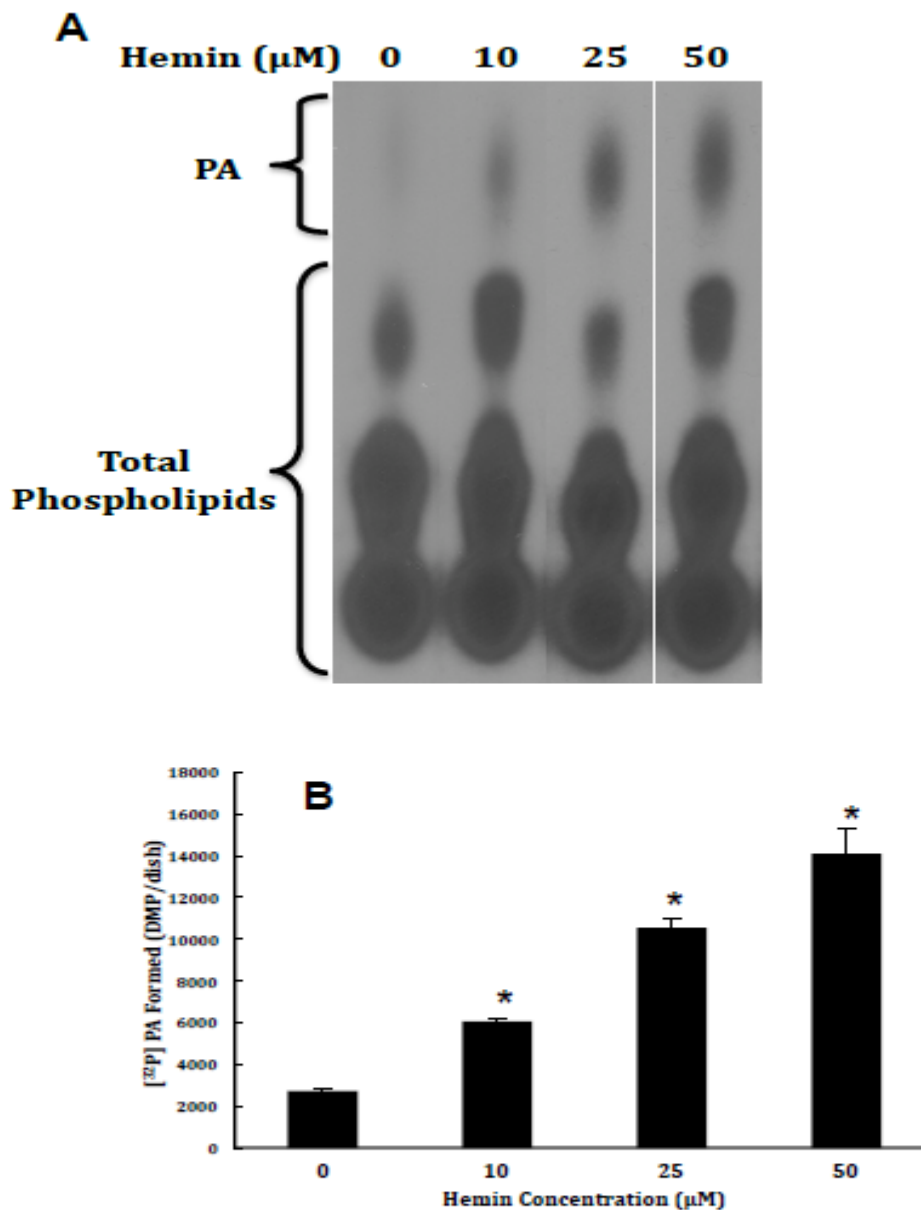


Figure 5: Hemin-induces formation of PLD-generated bioactive lipid, phosphatidic acid (PA) in a dose-dependent fashion in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with ^{32}P orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. Following ^{32}P orthophosphate labeling, cells were treated with MEM alone, MEM-containing hemin (10, 25, 50 μM) for 6 hours. At the end of the incubation period, ^{32}P PA formed was determined from the autoradiogram (A) and quantified from intensity (B). Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < .05$ as compared to cells treated with MEM alone.

Figure 6

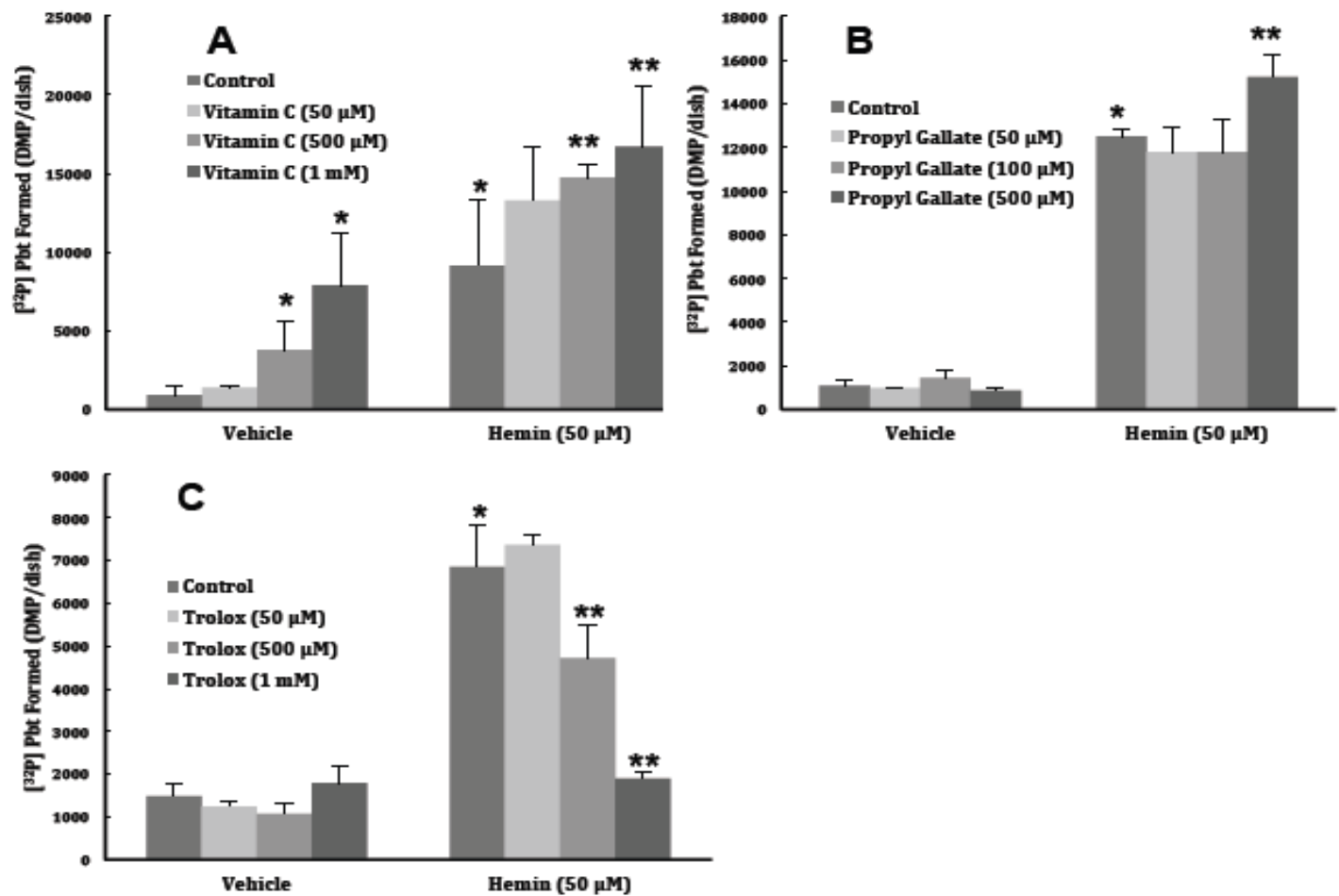


Figure 6: Antioxidants modulate hemin-induced phospholipase D (PLD) activation in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with [32 P]orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. (A) Following [32 P]orthophosphate labeling, cells were treated with minimal essential medium (MEM) alone, MEM containing Vitamin C (50 μM, 500 μM, 1 mM), MEM containing Hemin (50 μM), or MEM containing Vitamin C (50 μM, 500 μM, 1 mM) and Hemin (50 μM). (B) Cells were treated with minimal essential medium (MEM) alone, MEM containing Propyl Gallate (50, 100, 500 μM), MEM containing Hemin (50 μM), or MEM containing Propyl Gallate (50, 100, 500 μM) and Hemin (50 μM). (C) Cells were treated with minimal essential medium (MEM) alone, MEM containing Trolox (50 μM, 500 μM, 1 mM), MEM containing Hemin (50 μM), or MEM containing Trolox (50 μM, 500 μM, 1 mM) and Hemin (50 μM). All treatments were allowed to incubate for 6 hours. [32 P]phosphatidylbutanol ([32 P]Pbt) formation was determined and quantified as DPM/dish. Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with MEM alone.

Figure 7

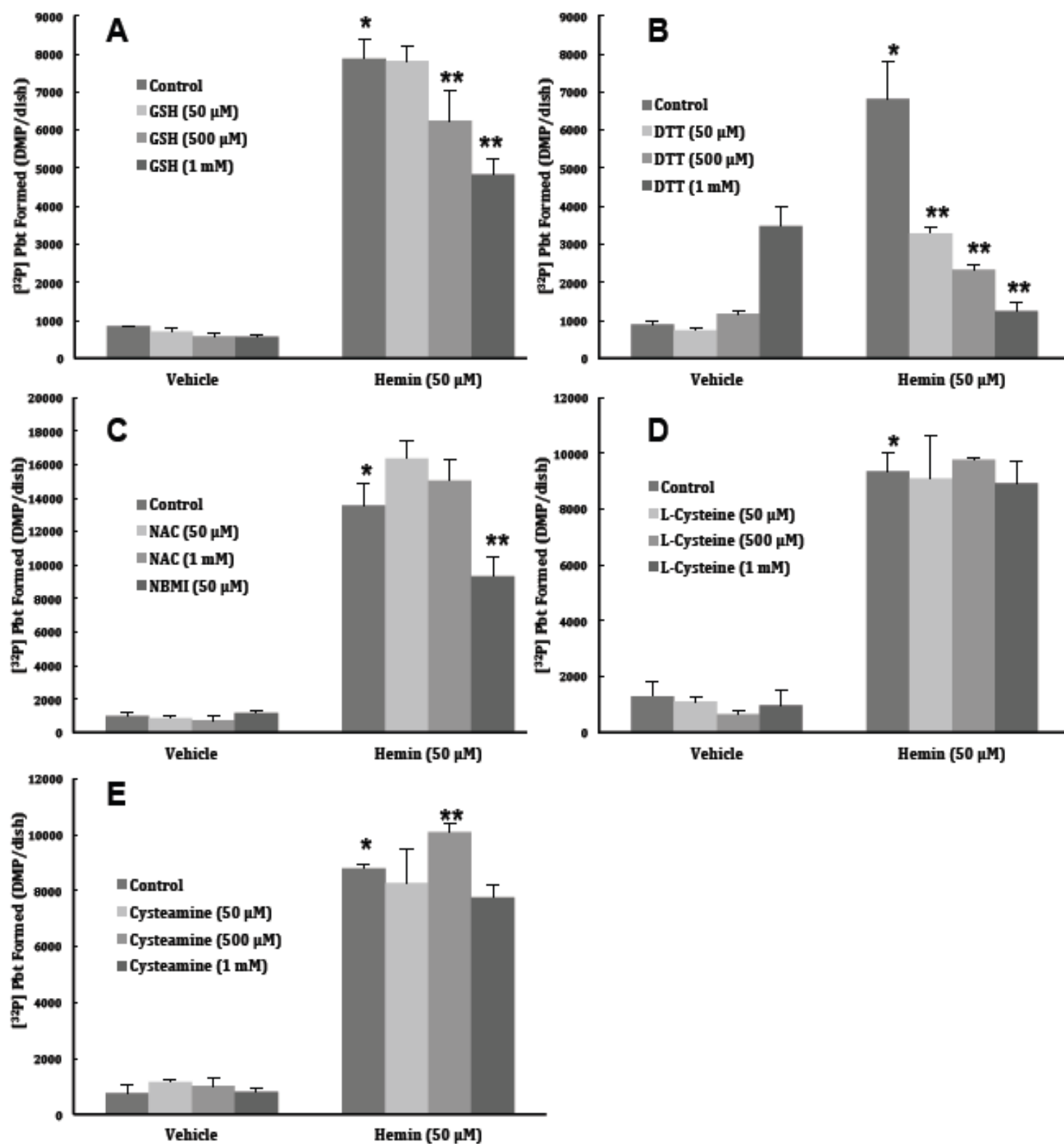


Figure 7: Sulfhydryl agents and thiol-protectants attenuate hemin-induced phospholipase D (PLD) activation in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with [32 P]orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. (A) Following [32 P]orthophosphate labeling, cells were treated with minimal essential medium (MEM) alone, MEM containing GSH (50 μ M, 500 μ M, 1 mM), MEM containing Hemin (50 μ M), or MEM containing GSH (50 μ M, 500 μ M, 1 mM) and Hemin (50 μ M). (B) Cells were treated with minimal essential medium (MEM) alone, MEM containing DTT (50 μ M, 500 μ M, 1 mM), MEM containing Hemin (50 μ M), or MEM containing DTT (50 μ M, 500 μ M, 1 mM) and Hemin (50 μ M). (C) Cells were treated with minimal essential medium (MEM) alone, MEM containing NAC (50 μ M, 1 mM), MEM containing NBMI (50 μ M), MEM containing NAC (50 μ M, 1 mM) and Hemin (50 μ M), or MEM containing NBMI (50 μ M) and Hemin (50 μ M). (D) Cells were treated with minimal essential medium (MEM) alone, MEM containing L-Cysteine (50 μ M, 500 μ M, 1 mM), MEM containing Hemin (50 μ M), or MEM containing L-Cysteine (50 μ M, 500 μ M, 1 mM) and Hemin (50 μ M). (E) Cells were treated with minimal essential medium (MEM) alone, MEM containing Cysteamine (50 μ M, 500 μ M, 1 mM), MEM containing Hemin (50 μ M), or MEM containing Cysteamine (50 μ M, 500 μ M, 1 mM) and Hemin (50 μ M). All treatments were allowed to incubate for 6 hours. [32 P]phosphatidylbutanol ([32 P]PBT) formation was determined and quantified as DPM/dish. Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with MEM alone.

Figure 8

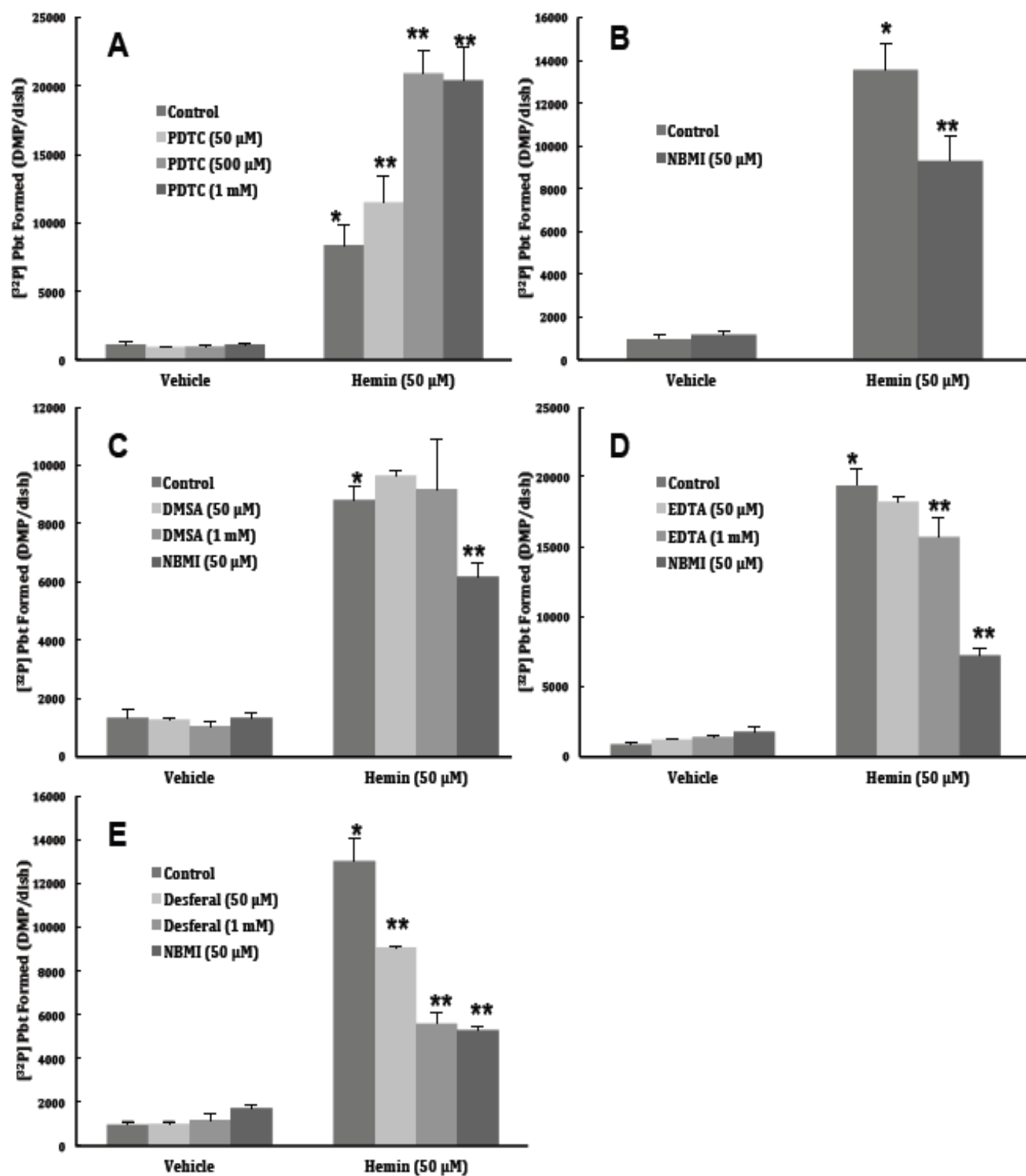


Figure 8: Heavy metal-chelating agents and novel thiol-redox chelator & antioxidant, NBMI, attenuate hemin-induced PLD activation in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with [32 P]orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. (A) Following [32 P]orthophosphate labeling, cells were treated with minimal essential medium (MEM) alone, MEM containing PDTC (50 μ M, 500 μ M, 1 mM), MEM containing Hemin (50 μ M), or MEM containing PDTC (50 μ M, 500 μ M, 1 mM) and Hemin (50 μ M). (B) Cells were treated with minimal essential medium (MEM) alone, MEM containing NBMI (50 μ M), MEM containing Hemin (50 μ M), or MEM containing NBMI (50 μ M) and Hemin (50 μ M). (C) Cells were treated with minimal essential medium (MEM) alone, MEM containing DMSA (50 μ M, 1 mM), MEM containing NBMI (50 μ M), MEM containing DMSA (50 μ M, 1 mM) and Hemin (50 μ M), or MEM containing NBMI (50 μ M) and hemin (50 μ M). (D) Cells were treated with minimal essential medium (MEM) alone, MEM containing EDTA (50 μ M, 1 mM), MEM containing NBMI (50 μ M), MEM containing EDTA (50 μ M, 1 mM) and Hemin (50 μ M), or MEM containing NBMI (50 μ M) and Hemin (50 μ M). (E) Cells were treated with minimal essential medium (MEM) alone, MEM containing Desferal (50 μ M, 1 mM), MEM containing NBMI (50 μ M), MEM containing Desferal (50 μ M, 1 mM) and Hemin (50 μ M), or MEM containing NBMI (50 μ M) and Hemin (50 μ M). All treatments were allowed to incubate for 6 hours. [32 P]phosphatidylbutanol ([32 P]Pbt) formation was determined and quantified as DPM/dish. Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with MEM alone.

Figure 9

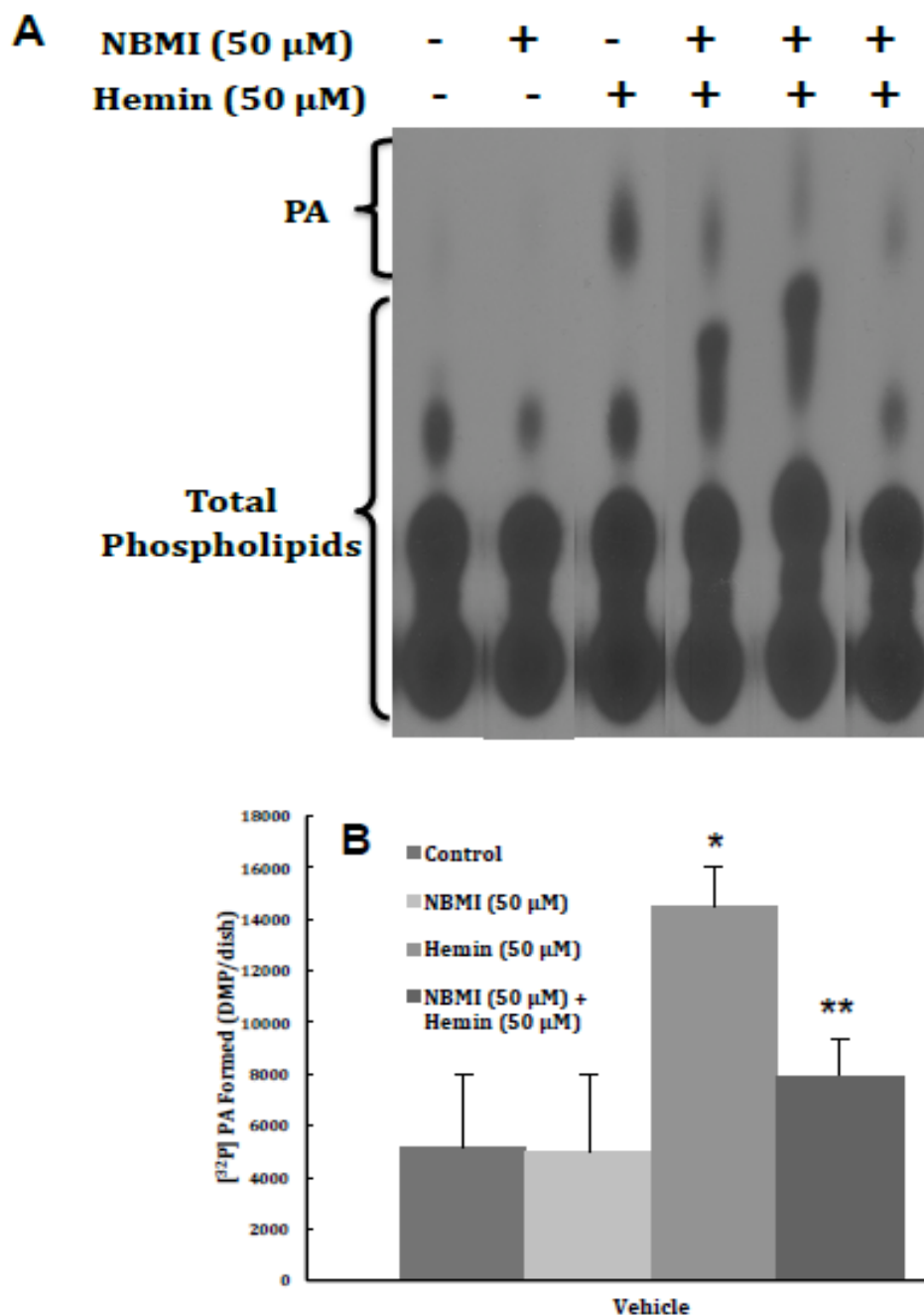


Figure 9: Novel lipophilic thiol-redox chelator and antioxidant drug (NBMI) attenuates Hemin-induced formation of PLD-generated bioactive lipid, phosphatidic acid (PA) in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with [³²P]orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. Following [³²P]orthophosphate labeling, cells were treated with MEM alone, MEM-containing hemin (50 μ M), MEM-containing NBMI (50 μ M), or MEM-containing hemin (50 μ M) + NBMI (50 μ M) for 6 hours. At the end of the incubation period, [³²P]PA formed was determined from the autoradiogram (A) and quantified from intensity (B). Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < .05$ as compared to cells treated with MEM alone.

Figure 10

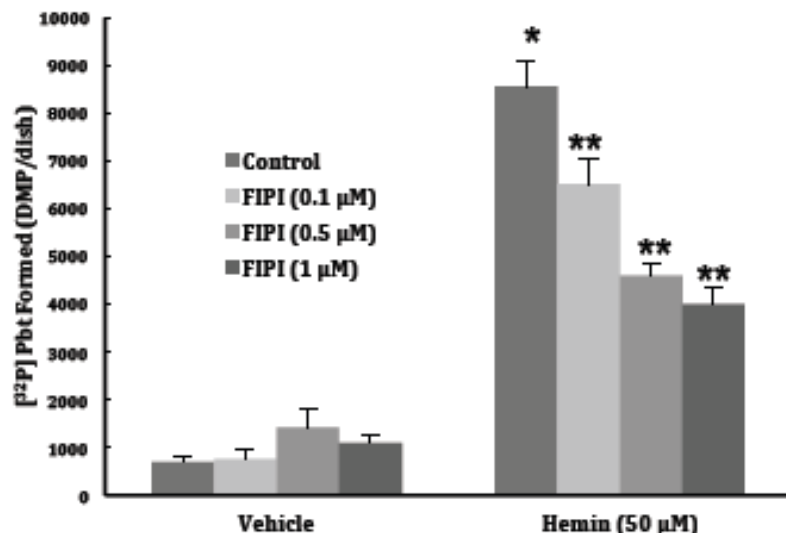


Figure 10: Phospholipase D (PLD)-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), attenuates Hemin-induced phospholipase D (PLD) activation in BLMVECs. BLMVECs (5×10^5 cells/35 mm dish) were labeled with [³²P]orthophosphate in phosphate-free Dulbecco-modified Eagle medium (DMEM) for 12 hours. Following [³²P]orthophosphate labeling, cells were treated with minimal essential medium (MEM) alone, MEM containing FIPI (0.1, 0.5, and 1 μM), MEM containing Hemin (50 μM), or MEM containing FIPI (0.1, 0.5, and 1 μM) and Hemin (50 μM) for 6 hours. [³²P]phosphatidylbutanol ([³²P]Pbt) formation was determined and quantified as DPM/dish. Data represent mean \pm standard deviation (SD) calculated from 3 independent experiments. *Significantly different at $P < 0.05$ as compared to cells treated with MEM alone.

Figure 11

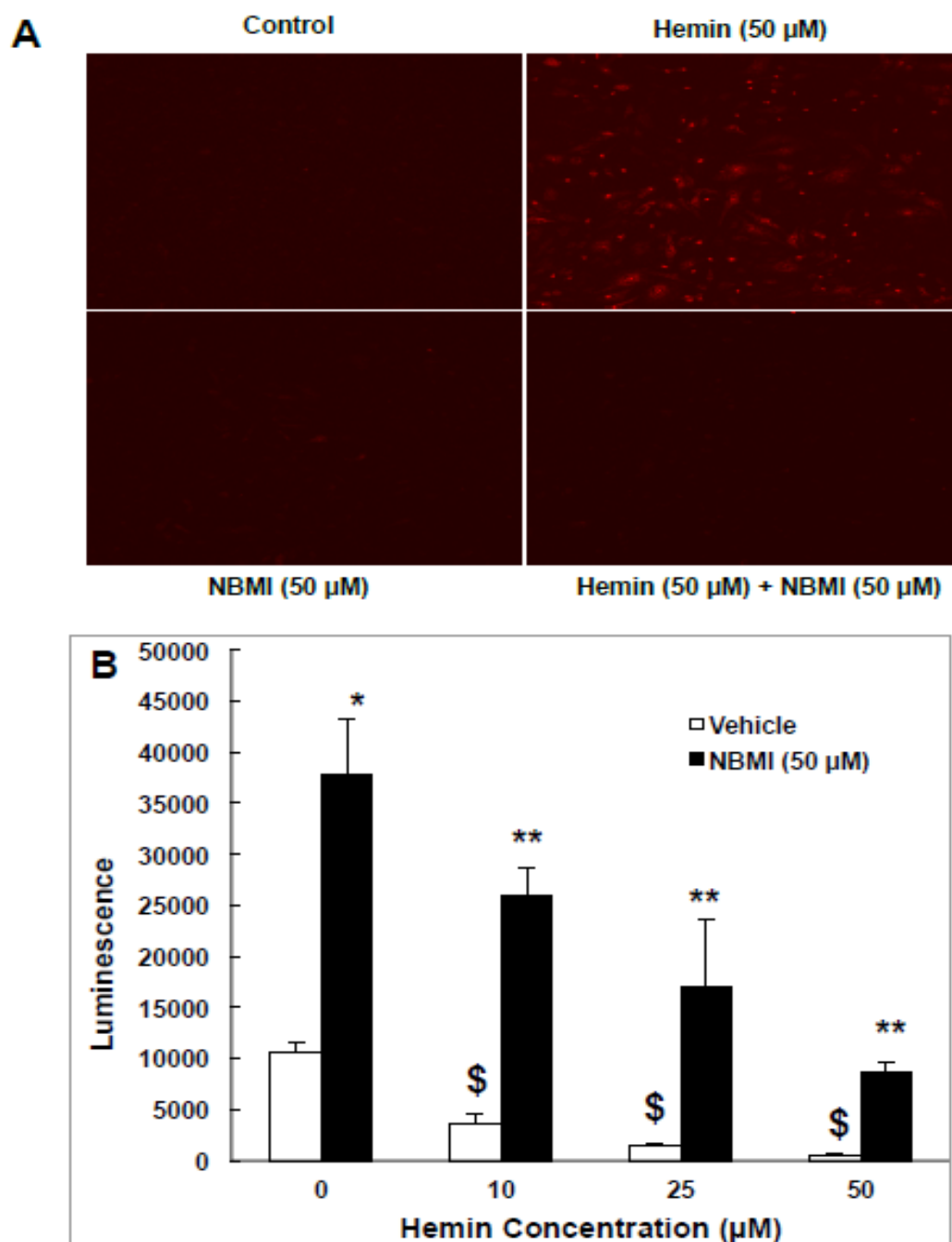


Figure 11: NBMI protects against hemin-induced ROS formation and loss of GSH in BLMVECs. BLMVECs grown to 95% confluence in 35-mm dishes were treated with MEM alone, MEM-containing hemin (50 μ M), MEM-containing NBMI (50 μ M), or MEM-containing hemin (50 μ M) + NBMI (50 μ M) for 1 h and incubated at 37°C in a humidified environment of 5% CO₂-95% air (A). At the end of treatment, images were captured digitally as described in *materials and methods*.

BLMVECs (5×10^5 cells/35 mm dish) were pretreated with minimal essential medium (MEM) alone, MEM containing NBMI 50 μ M, for 2 hours. Following pre-treatment, BLMVECs were treated with MEM alone, MEM containing hemin (10, 25, 50 μ M), or MEM containing hemin (10, 25, 50 μ M) and NBMI (50 μ M), (B) for 4 hours. At the end of the incubation period, the intracellular soluble thiol (glutathione [GSH]) concentrations were determined. Data represent mean \pm standard deviation (SD) calculated from 3 independent

Figure 12

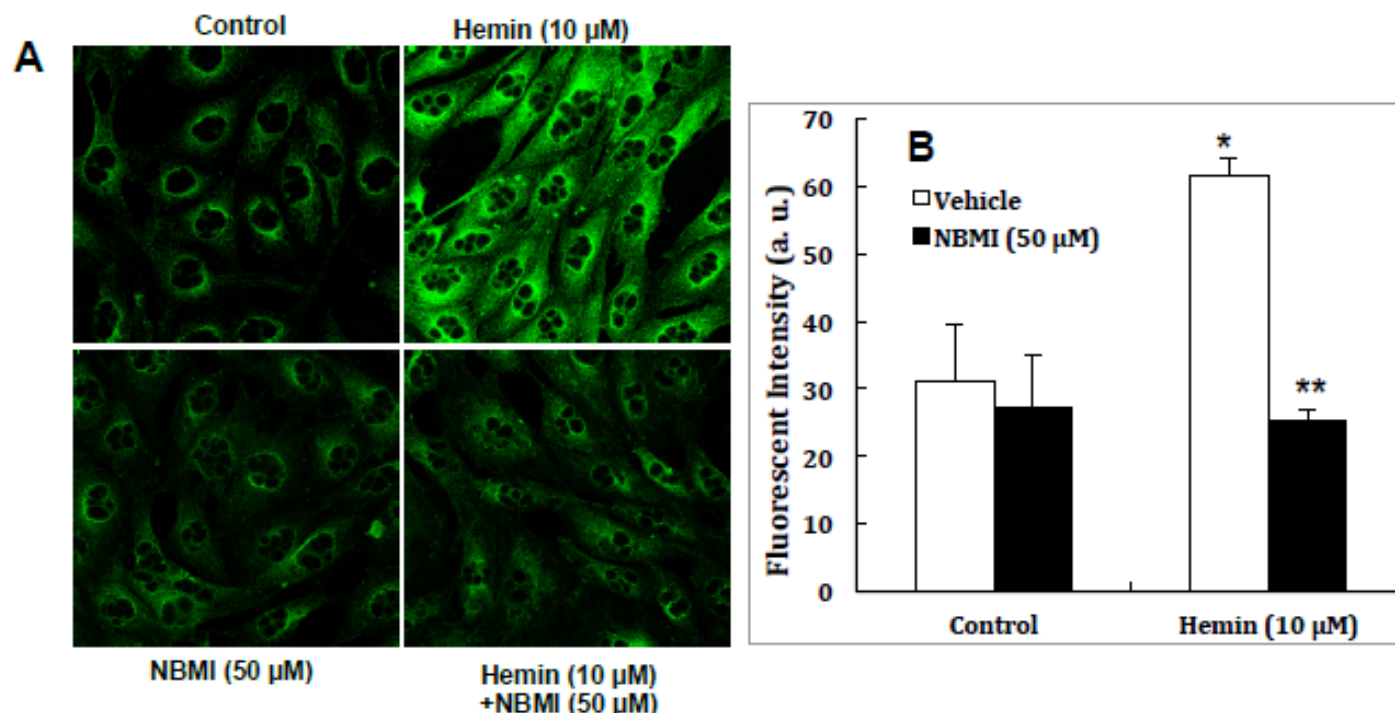


Figure 12: NBMI Protects against hemin-induced lipid peroxidation (formation of 4-hydroxy-2-nonenal, 4-HNE) in BLMVECs. BLMVECs grown to 95% confluence in 35-mm dishes were treated with MEM alone, MEM-containing hemin (10 μ M), MEM-containing NBMI (50 μ M), or MEM-containing hemin (10 μ M) + NBMI (50 μ M) for 1 h and incubated at 37 $^{\circ}$ C in a humidified environment of 5% CO_2 -95% air. At the end of treatment, cells were fixed, stained for 4-HNE (A, B), and images were captured digitally as described in *materials and methods*. The fluorescence intensities were measured as described under Materials and Methods. Data represent mean \pm SD calculated from three independent experiments. *Significantly different at $p < 0.05$ as compared to cells treated with MEM alone. **Significantly different at $p < 0.05$ as compared to cells treated with MEM containing hemin alone.

Figure 13

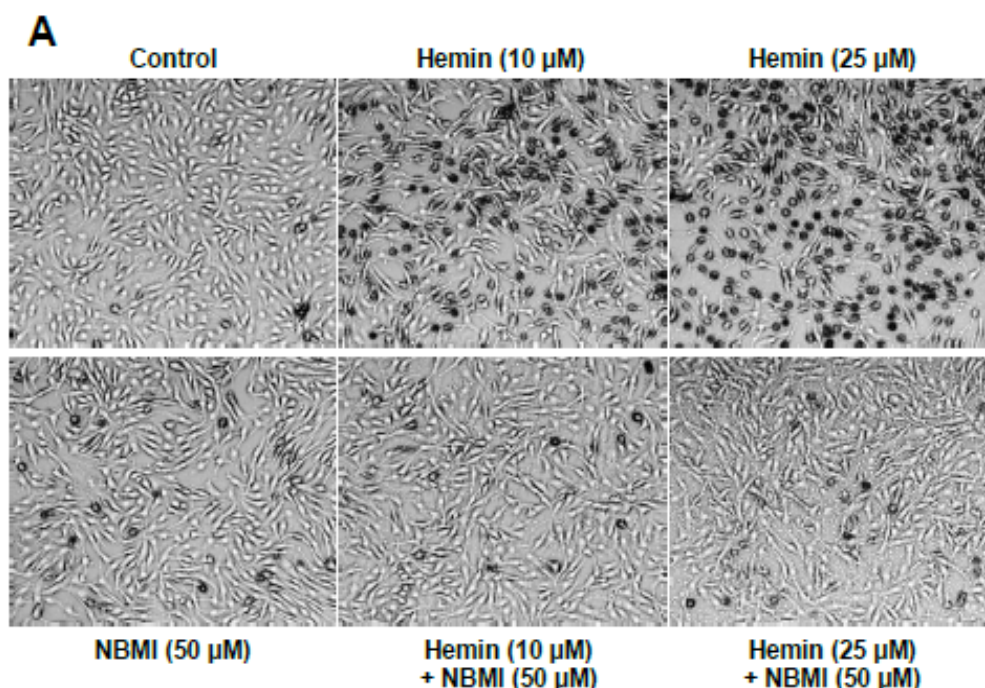


Figure13A: NBMI protects against hemin-induced alterations in endothelial cell morphology in BLMVECs. BLMVECs grown to 95% confluence in 12-well culture plates were treated with MEM alone, MEM-containing hemin (50 μ M), MEM-containing NBMI (50 μ M), or MEM-containing hemin (10, 25 μ M) + NBMI (50 μ M) for 4 h and incubated at 37°C in a humidified environment of 5% CO₂-95% air. At the end of the incubation period, the cell morphology was examined under light microscope (as an index of EC monolayer disruption and cytotoxicity) as described under the section “Materials and Methods”. Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

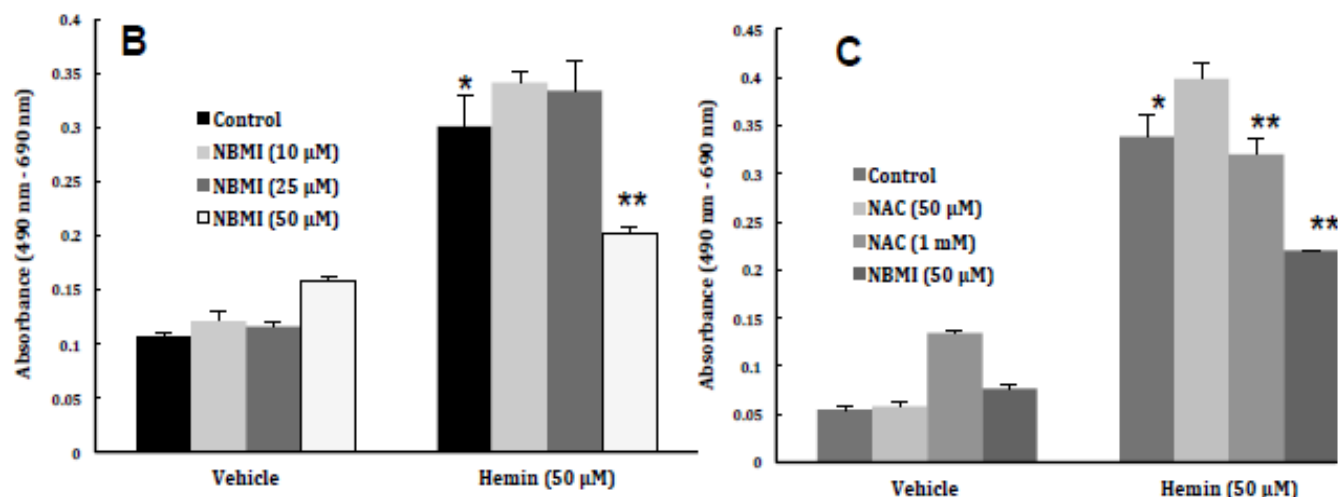


Figure 13B, C: NBMI protects against Hemin-induced LDH release (cytotoxicity) in BLMVECs. (B) BLMVECs grown to 95% confluence in 24-well culture plates were treated with minimal essential medium (MEM) alone, MEM containing NBMI (10, 25, 50 μ M), MEM-containing Hemin (50 μ M), or MEM-containing NBMI (10, 25, 50 μ M) and Hemin (50 μ M). (C) BLMVECs grown to 95% confluence in 24-well culture plates were treated with MEM alone, MEM containing NAC (50 μ M, 1 mM), MEM containing NBMI (50 μ M), MEM containing NAC (50 μ M, 1 mM) and Hemin (50 μ M), or MEM containing NBMI (50 μ M) and Hemin (50 μ M). All treatments were incubated at 37°C in a humidified environment of 5% CO₂-95% air for 6 hours. Release of LDH (as an index of cytotoxicity) was determined spectrophotometrically as described in *materials and methods*.

Figure 14

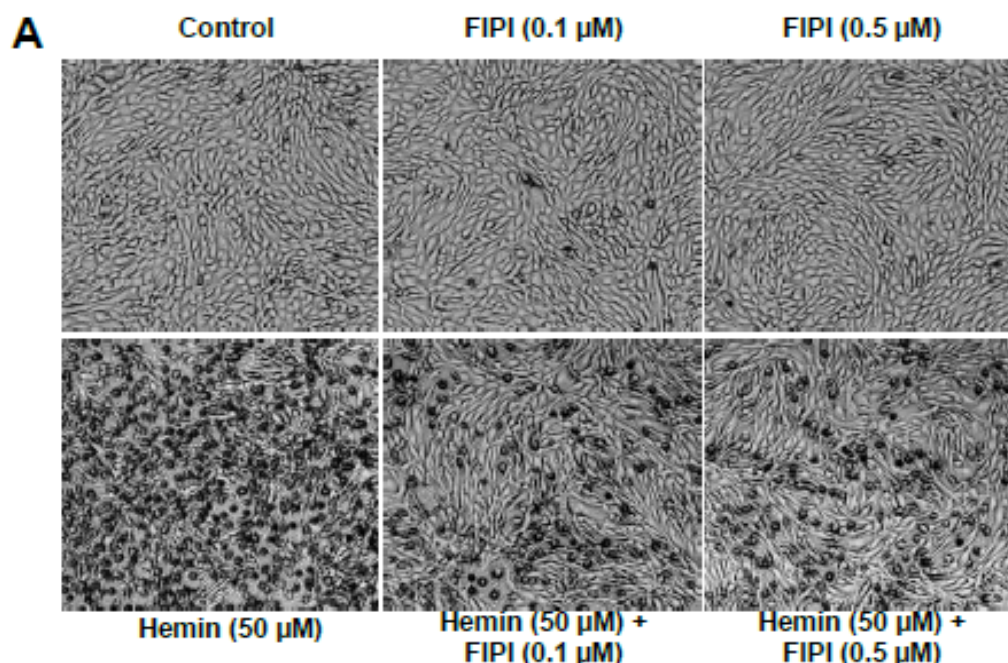


Figure 14A: FIPI protects against hemin-induced alterations in endothelial cell morphology. BLMVECs grown to 95% confluence in 12-well culture plates were treated with MEM alone, MEM-containing hemin (50 μ M), MEM-containing FIPI (0.1, 0.5 μ M), or MEM-containing hemin (50 μ M) + FIPI (0.1, 0.5 μ M) for 4 h and incubated at 37°C in a humidified environment of 5% CO₂-95% air. At the end of incubation, cells were examined under light microscope at 10X magnification as described in *materials and methods*. (as an index of EC monolayer disruption and cytotoxicity) as described under the section "Materials and Methods". Each micrograph is a representative picture obtained from three independent experiments conducted under identical conditions.

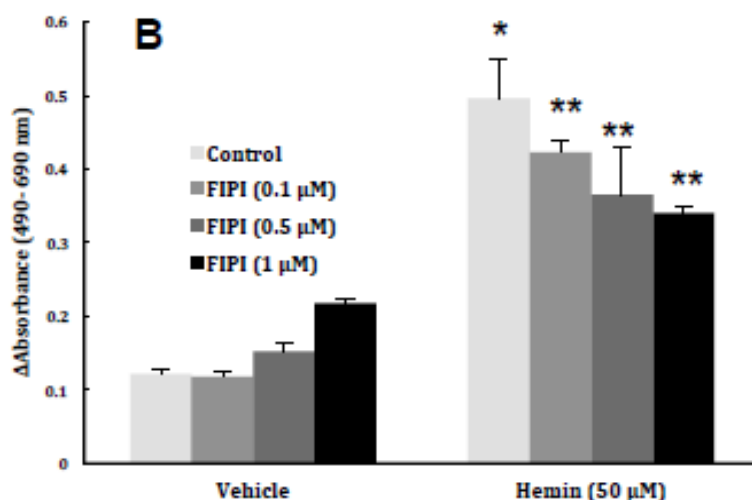
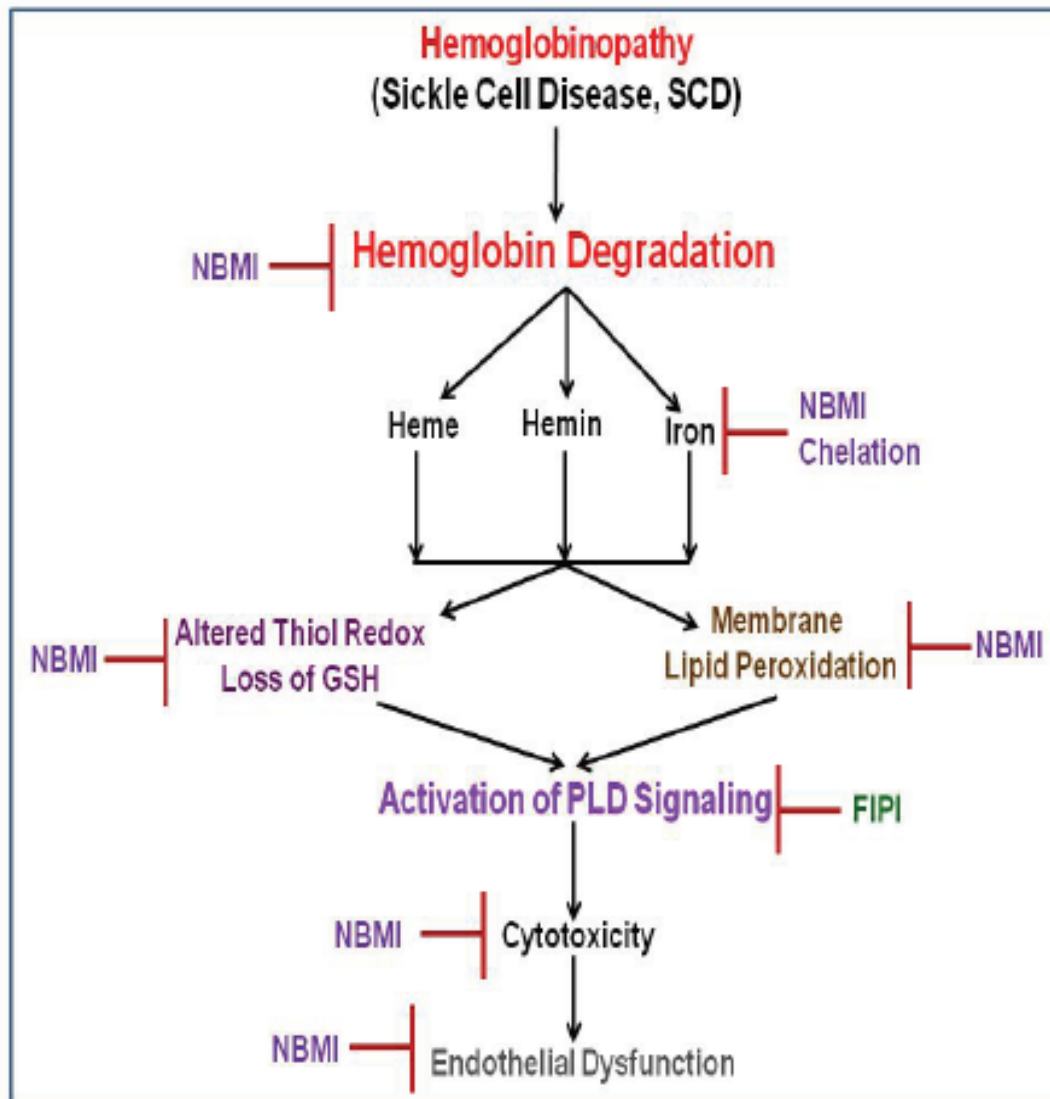


Figure 14B: Phospholipase D (PLD)-specific inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), attenuates hemin-induced LDH release by endothelial cells. BLMVECs grown to 95% confluence in 24-well culture plates were treated with minimal essential medium (MEM) alone, MEM containing FIPI (0.1, 0.5, 1 μ M), MEM-containing Hemin (50 μ M), or MEM-containing NBMI (0.1, 0.5, 1 μ M) and Hemin (50 μ M) and incubated at 37°C in a humidified environment of 5% CO₂-95% air for 6 hours. Release of LDH (as an index of cytotoxicity) was determined spectrophotometrically as described in *materials and methods*.

Figure 15



Schema: Proposed mechanism of hemin-induced activation of phospholipaseD (PLD) - mediated lipid signaling through oxidant production and oxidative stress leading to cytotoxicity in BLMVECs. The novel lipophilic thiol-redox chelator and antioxidant drug protected against the hemin-induced cytotoxicity in BLMVECs through attenuation of ROS production, loss of GSH status, and PLD activation. The protection of hemin induced PLD activation and cytotoxicity in BLMVECs by the PLD-specific inhibitor, FIPI, confirmed the role of PLD-mediated lipid signaling and PA in the hemin-induced cytotoxicity in BLMVECs.